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PhD Thesis

Functional Feed Additives for Animal Health

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This journey made me face the most severe challenges of my life. I dedicated all myself to achieve everyday a better result and learn from each situation.
However, I also had to face one of the darkest moments of this era: the pandemic that completely cancelled my life. All what I loved to do was banned and the people who I wanted to live with were separated from me…
Nobody was ready for this.

Nevertheless, it is in the darkest moments where we can find the light.

During these years I lived through difficult times, making great sacrifices and facing my fears.
Still, not even for one second, I stopped chasing my dreams, bringing out all the strength and passion that I feel inside.
All that you will find in this thesis is the result of discipline, commitment and hours and hours of beating on my craft.
During this period, the heart pushed the mind to overtake each obstacle when it seemed impossible…

I want to dedicate this page to people who believe that impossible does not exist. To all person which knows that even if today you have been defeated, tomorrow you will have a new opportunity because the failure is only for who quits.

I want to dedicate this page to all the people that every day wake up to make the difference…

I also would like to recognize merits to who always believed in me, leading myself to achieve results that I have never imagined.

To my teammates who have been my family, making me a better person and demonstrated that together we can become unstoppable.
To my tutor, Prof. Luciana Rossi, a person that changed my life, pushing me to develop my strongest skills ever and let shine my light by feeding the flame that I feel inside.

Luciana is the person who made possible everything that you will find described in the next pages. She realized all of this combining passion, intelligence and the most powerful strength existing in life, which is able to move mountains and cross oceans: love.

This is one of the greatest lessons of my life that I will never forget.

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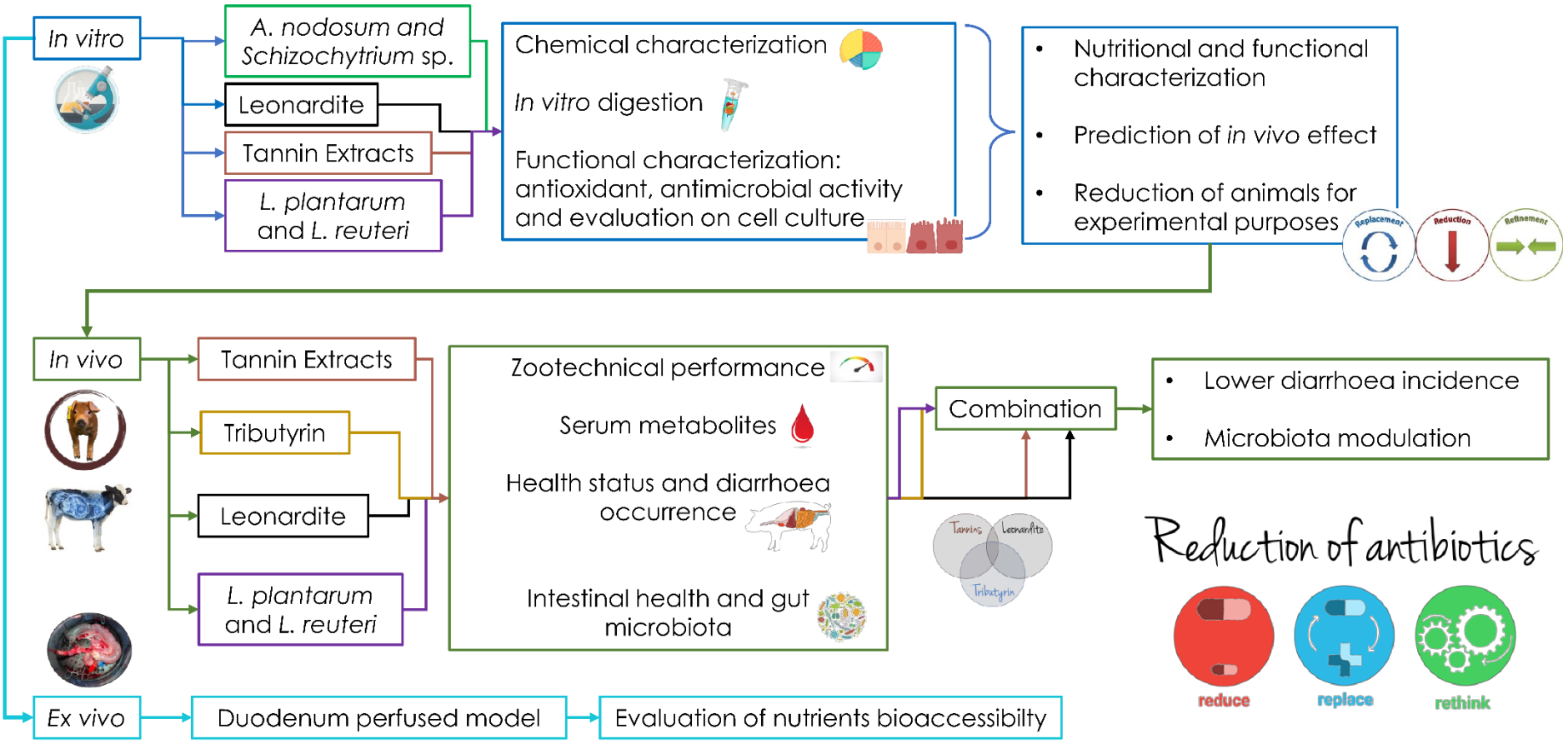
Abstract

World population growth is leading to an increase of the of food from animal origin demand. For the future of the planet and food-production system sustainability some important issues required to be faced. Antimicrobial resistance is one of the most important global concerns cause of thousands of deaths per year and livestock can contribute to the antimicrobial resistance spreading. In livestock, the highest use of antibiotic treatments is recorded in young animals, due to their immature immune system, and the presence of several stress sources that predispose young animals to develop multifactorial diseases, particularly from gastrointestinal origin, that causes important economic losses and antibiotic use. Nutrition is no longer considered as science that only satisfies nutritional requirements but is considered fundamental for its functionality through the supplementation of ingredients and additives which contain bioactive molecules that positively impact on health. Functional feed ingredients and additives possess bioactive characteristics, such as antibacterial and antioxidant activities, capable of contrasting infections and improving animal health or a particular organic function. Preserving intestinal health decreases the incidence of pathologies, optimises digestive processes, and promotes animal performance thus resulting in higher profitability for the farmers. The aim of the following project was to evaluate innovative functional feed additives for animal nutrition, in order to increase animal health, performance and reduce the use of antibiotics in farm animals. The attention was focused on the evaluation of algae, tannins extract, leonardite, tributyrin and probiotics as functional feed additives alternative to antibiotics. For reaching this goal different *in vitro, in vivo* and *ex vivo* approaches have been used. *In vitro* methods allowed to characterize functional feed additives for their antimicrobial and antioxidants effects and cell culture was useful to predict their interaction with the intestinal epithelium. *In vivo* zootechnical trials were conducted on young animals to assess dietary supplementation of functional additives on growth performance, prevention of gastroenteric disorders, metabolic status and microbiota modulation. Obtained results highlighted that functional additives were characterized by bioactive properties such as antioxidant and antimicrobial effects. Their supplementation showed a plethora of positive impacts including higher zootechnical performance, decreased diarrhoea occurrence, improved health status and microbiota modulation. A combined effect was also assessed in order to evaluate a possible synergistic of different additives. The combination resulted in a positive effect for diarrhoea prevention with a slight modulation for the gut microbial environment. Even if feed additives displayed different bioactivities *in vitro*, they did not always reveal a boosting effect on growth performance *in vivo* resulting less evident for farmer

perception. However, the reduction of gastrointestinal disorders incidence could be considered the most interesting characteristic of functional feed additives which have shown to possibly reduce pathologies occurrence thus improving farm profitability.
Lastly, due to the needing to decrease animal for experimental purposes an innovative *ex vivo* intestinal perfusion model was developed in order to study nutrient bioaccessibility for obtaining reliable science-based data in line with the 3R principles.
In conclusion, tested compounds can not substitute the therapeutic treatment of antibiotics. However, the utilization dietary approaches supplementing functional feed additives can significantly contribute for decreasing pathology occurrence, boosting animal health and performance thus the antibiotics use. In line with the One Health principles, antibiotics reduction is a key point for the future sustainable development on the planet. The use of functional feed additives can be considered as interesting alternative to antibiotics due to their ability to prevent multifactorial disorders and thus reducing the antibiotic treatments in livestock farming.

Graphical Abstract

Functional Feed Additives for Animal Health



1 Introduction

1.1 General Scenario

The World Health Organization (WHO) estimated that by 2030, the world's population is projected to increase again, by slightly more than one billion to 8.6 billion, and to increase further to 9.8 billion in 2050. Global milk production in 2030 will be 33 percent above the 2015–2017 baseline. Production growth in developing countries will stem from a combined increase in dairy herds of about 1.2 percent per year, and a yield increase of about 1.0 percent per year; while in developed countries growth will mainly come from improved yields. By 2030, India will have outpaced the European Union (Member Organization) to become the largest milk producer and will, together with Pakistan, account for nearly one-third of world milk production. Global meat production is projected to be 19 percent higher in 2030 relative to the 2015–2017 base period. While the largest producers (Brazil, China, the European Union (Member Organization) and the United States of America) will continue to dominate meat production, developing countries are expected to account for almost all of the total increase (FAO, 2018). In light of this, to sustain the world and population development it is necessary to face critical challenges for the planet's health trough an integrated approach.

The term "One Health" was firstly introduced during the emergence of severe acute respiratory disease (SARS) and the spread of avian influenza H5N1. One Health is commonly defined as the collaborative, multisectoral, and transdisciplinary approach working at the local, regional and national, and global levels with the goal of achieving optimal health outcomes recognising the interconnection between human, animals, pants and their shared environment. Furthermore, One Health recognizes that the health of humans, animals and ecosystems are interconnected. It involves a coordinated, collaborative, multidisciplinary and cross-sectoral approaches to address potential or existing risks that originate at the animal-human-ecosystems interface (Mackenzie and Jeggo, 2019).

In line with the following concept, United Nations adopted the 2030 Agenda setting 17 ambitious goals to promote health and wellbeing. The sustainable development goals are a call to action to end poverty and inequality, protect the planet, and guarantee health, justice, and prosperity.

The European Green Deal is the European strategy for achieving a transformational change. The bond among healthy people, healthy society and healthy planet are the core of European Green Deal. It has been meant to stimulate the economy, health and life quality enhancement protecting the nature. The European agricultural system is a

global standard in terms of security, safety, nutrition, and quality. Now it has to become a global standard for the sustainability guiding the transition towards a competitive sustainability from farm to fork. Following these principles, Italy aims to achieve an entire sustainable food-chain through the national recovery and resilience plan (Piano Nazionale di Ripresa e Resilienza - PNRR).

The European Feed Manufacturers' Federation (FEFAC) included five ambitions for the feed industry for 2030: i) contribute to climate-neutral livestock and aquaculture production through feed; ii) foster sustainable food system through increased resource and nutrient efficiency; iii) promote responsible sourcing practices; iv) contribute to improving farm animal health and welfare, v) enhance socio-economic and the livestock and aquaculture sectors' resilience. In particular for contribute to improve farm animal health and welfare antibiotic reduction is a pivotal point of the ambition and many countries such as Belgium, France, Denmark, Italy, Germany, United Kingdom and Netherlands adopted a position on responsible use of veterinary medicine and some of them target to achieve the 0% of antibiotic-medicated feed production for 2030 (FEFAC, 2020).

In line with all the sustainability plans adopted from world countries, a key point for the future development of livestock system is to face the raising issue of antimicrobial resistance. It has become fundamental to cope with this concern to ensure the future of humans, animals and the environment.

1.2 Antibiotic resistance

Even if during the last years the mediatic attention have been moved on the pandemic emergency, the antimicrobial resistance (AMR) is still an increasing issue on this planet. Antimicrobials are drugs used to prevent and treat infections in humans, animals, and plants. They comprehend antibiotics, antivirals, antifungals and antiparasitic agents. AMR occurs when bacteria, viruses, fungi and parasites no longer respond to medicines (WHO, 2015). The direct consequences of infection by resistant microorganisms can be serious, including severe illnesses, raised mortality, increased direct and indirect costs. Antimicrobial resistance is a worldwide problem affecting all areas of health with an effect on humans, animals and the environment.

Discovery of antibiotics in early 1900s completely changed the human and veterinary medicine by saving millions of lives. Antibiotics are complex compounds capable of inhibiting the growth of bacteria by a various mechanism of action which includes, cell membranes alteration, cell wall synthesis inhibition, antimetabolic activity, nucleic acids synthesis inhibition, protein synthesis alteration (Pancu et al., 2021). Antibiotic resistance can be included in the broader term of antimicrobial resistance, and it occurs

when bacteria develop the ability to grow in presence of antibiotics. Antibiotic resistance is one of the most important issues for the public health in Europe and all over the world. The World Health Organization estimated that in 2022 the European Union/European Economic Space registered more than 670,000 infections per year from antibiotic resistant bacteria, and around 33,000 deaths as direct consequence of the problem. It was estimated that an integrated strategy of intervention comprehensive of antibiotics management programs, hygienic improvement, mass media communication operations and rapid diagnostic tests implementation can potentially prevent 27,000 deaths per year in the European Union. Beside saving human lives, a similar result could lead to save around 1.4 billion €/year for the public health in Europe (WHO, 2022). All the most important world agencies (WHO, FAO, OIE, UNEP and EFSA) are intensively working to minimize the spread of antimicrobial resistance. Today there is the necessity to develop innovative strategies that can decrease the usage of antibiotic drugs (Cormican et al., 2017; Tiedje, 2019).
Antibiotic-resistant bacteria can arise by using antibiotics in human and animals, since they are excreted and spread in the environment as hazardous microorganisms (Figure 1) (Dafale et al., 2020).

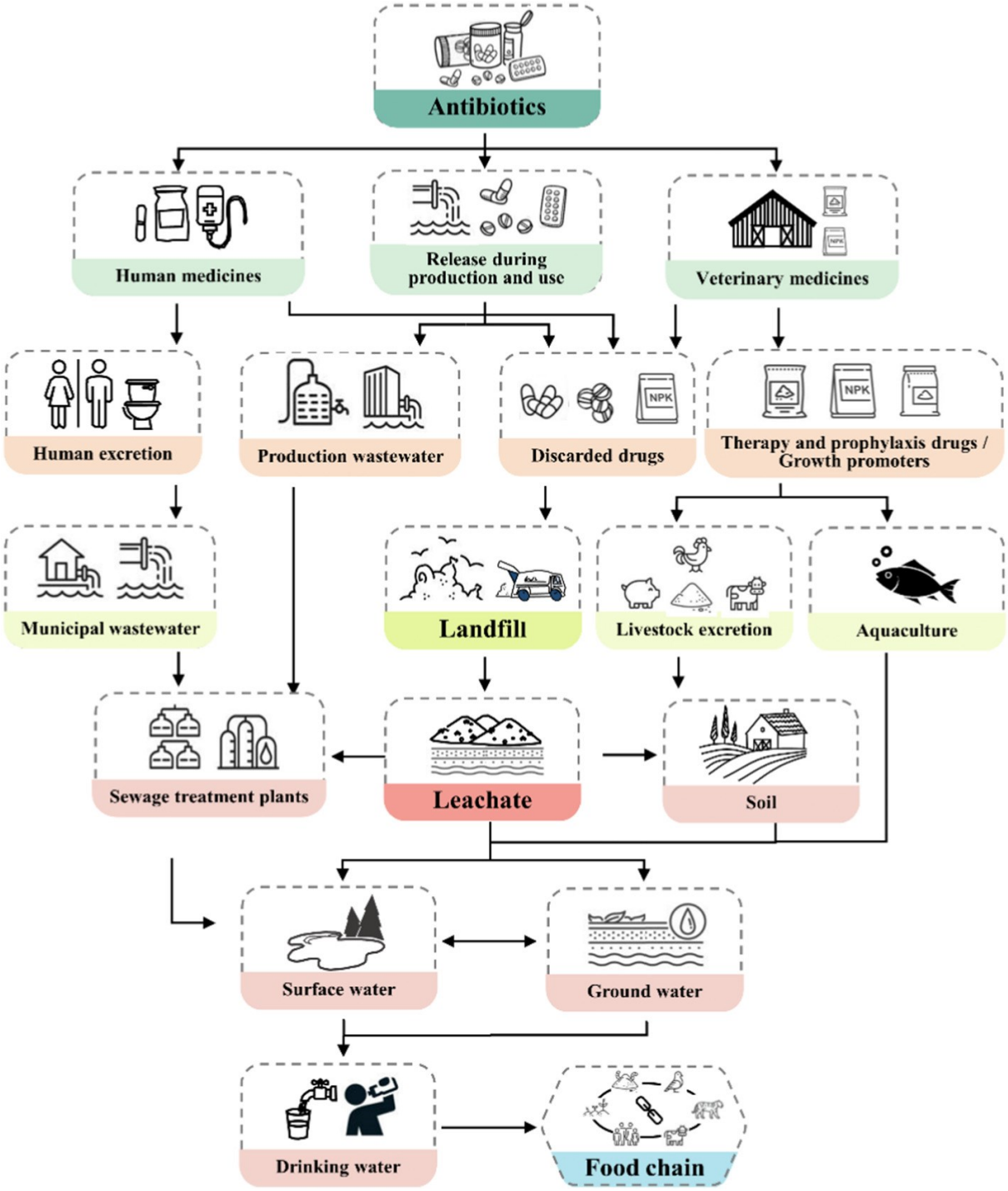


Figure 1. The pathways of antibiotic resistance spreading in the environment (Zhang et al., 2022a).

Through the Darwinian selection, microorganisms coped with antimicrobial selection pressure, due to antimicrobial use and overuse, enhancing their fitness developing and transferring resistance genes (McEwen and Collignon, 2018). The widest part of emerging diseases crossed the trans-boundaries of species becoming zoonotic. Researchers hypothesise that pathogens evolve in different ecosystems using any available biological host. The most common hosts are animals. In particular, the gut microbiota is composed by various microbial communities that are directly linked

with the health of their host (Purohit, 2018). The introduction of resistant bacteria in the intestinal microbiota can alter community and structure by transferring resistance genes to other pathogens and non pathogens. These microorganisms can evolve or find a route to be transferred from animal to animal or from animals to humans, and environment, spreading the resistance genes. The transfer of antibiotic-resistance genes is associated with mobile genetic elements (MGEs) such as transposons, plasmids, and genomic islands that can be exchanged to other bacteria (Figure 2).

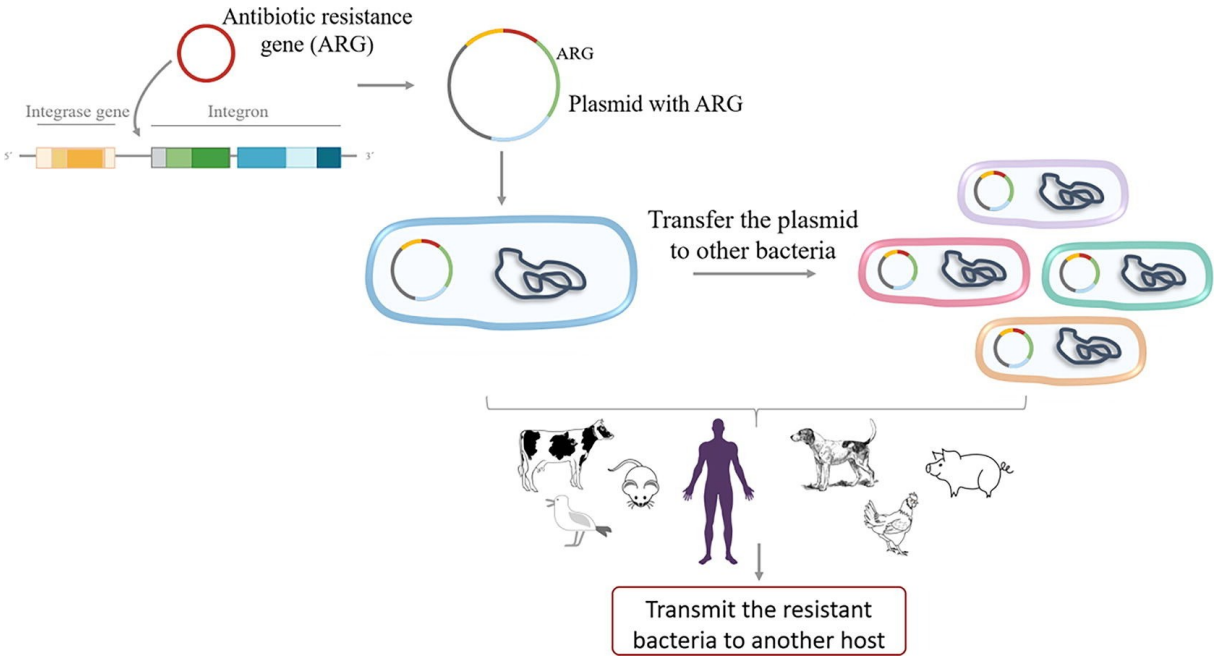


Figure 2. Antibiotic resistance transmission scheme (Gil-Gil et al., 2021).

Antibiotics are used in the livestock system to treat bacterial infections that occur during animals' production cycle. Until 2006, antibiotics have been used as growth promoters in farm animals to raise their performance. This practice brought to an uncontrolled usage of antimicrobial drugs applying a selective pressure, fastening the development of resistant bacteria, and spreading them into the environment (Parmar et al., 2018). In addition, the agricultural practices, such as the use of manure, spread antibiotic residues in different ecological compartments such as soil and water. Even if many studies have been conducted on zoonotic pathogens, a proper regulation and management of antibiotic drugs along with judicious use is still lacking.

Nowadays the health is considered as one unique interconnection among humans, animals and environment under the definition of One Health. To cope with the raising concern of antibiotic resistance a multidimensional approach, following One Health principles by managing all factors involved in zoonotic infections. The following approach includes multidisciplinary fields of researchers, policy makers, and leaders at a regional, local, national and international levels (Binot et al., 2015). One Health

connects multiple sectors for understanding the antimicrobial resistance problem to developing efficient solutions, suitable guidelines for drug utilization and raise the awareness of the risk of this problem.

For a multifaceted problem it is required an holistic approach considering the i) regulations for the use of different classes of antimicrobial and their critical importance; ii) understanding of the resistance dissemination into the environment to study the propagation pathways of antimicrobial resistance from host animals and humans; iii) develop innovative solutions to quantify the resistance and manage the presence of resistance genes in livestock; iv) effective communication to raise the consumer awareness regarding the risk associated with antimicrobial resistance spreading from food production and other sectors.

Considering the population growth, the rising of animal food products demand and the increasing of environmental pollution and antimicrobial resistance, the scientific community is driven to find new ways to produce sustainable food and feed (EFSA, 2015; FAO, 2017).

In animal farming, the neonatal and weaning phases are a critical where different multifactorial diseases can affect animals due to immune system immaturity and the progressive lowering of maternal immunity. Some of the most important criticisms in these phases are bound to gastrointestinal disorders. In swine farming, one of the most common diseases after weaning is the post-weaning diarrhoea (PWD). In cattle breeding the corresponding problem generally impact during the pre-weaning phase with the occurrence of the neonatal calf diarrhoea (NCD). These disorders often require the use of antibiotics to limit their detrimental effect on animal health and welfare. During last years, antibiotics use has been importantly decreased in livestock and the European Union prompted regulations to ban antibiotic treatments as growth promoters (Regulation EC 1831/2003), prophylactic treatment and recently promoted a conscious use of antibiotic drugs in livestock (Regulation EU 6/2019).

1.3 Gastrointestinal Disorders in Young Animals

Young animals are at the basis of the production system in animal farming. In this phase, they are particularly susceptible to develop different multifactorial pathologies due to several stressors that they have to cope with and the immaturity of their immune system. The gut is not only the site of nutrient absorption, it is also i) the first access for the most of pathogen present in the farm environment; ii) the niche of microbiota community; iii) the location of several immune systems components. Gastrointestinal disorders are cause of important economic losses for farmers and the environmental spread and contamination from pathogens. In intensive farming

gastrointestinal disorders are the first cause of antibiotic use thus leading to reduced performance, cost of treatments, impaired intestinal eubiosis (dysbiosis). Eubiosis is a state of microbial composition in which microbial groups and functions are well balanced and associated with a healthy state. Dysbiosis is the alteration in the relative abundance of microbial groups or functions that cause an imbalance, generally leading to a detrimental change in health (Figure 3, Figure 4). Microbes are not inherently mutualistic or pathogen but navigate between shades of mutualism and parasitism. The immune system is not designed to discriminate between mutualist or pathogens, but merely to react to signals, including Microbe-Associated Molecular Patterns and antigens. The nature of the immune response is not purely regulatory or inflammatory, but more generally adjusts to the nature of the trigger it faces, such as a spring that is pulled by the intensity of the microbial challenge (Eberl, 2010; Avelar Rodriguez et al., 2019).

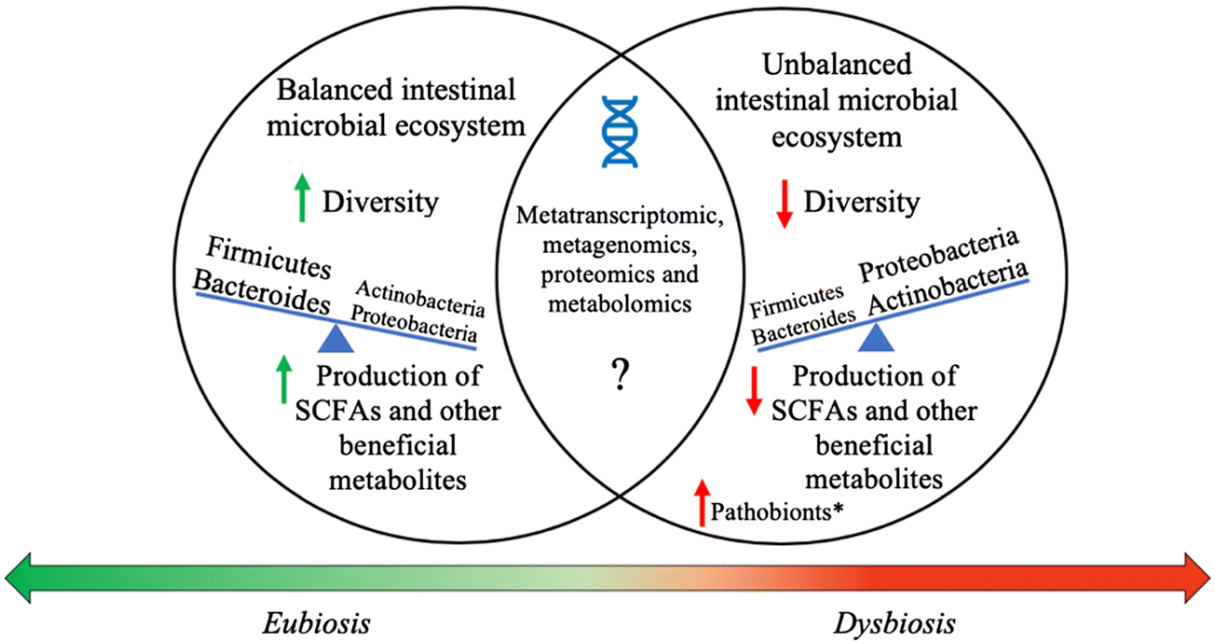


Figure 3. Schematic representation of the general current understating of intestinal eubiosis and dysbiosis (Avelar Rodriguez et al., 2019).

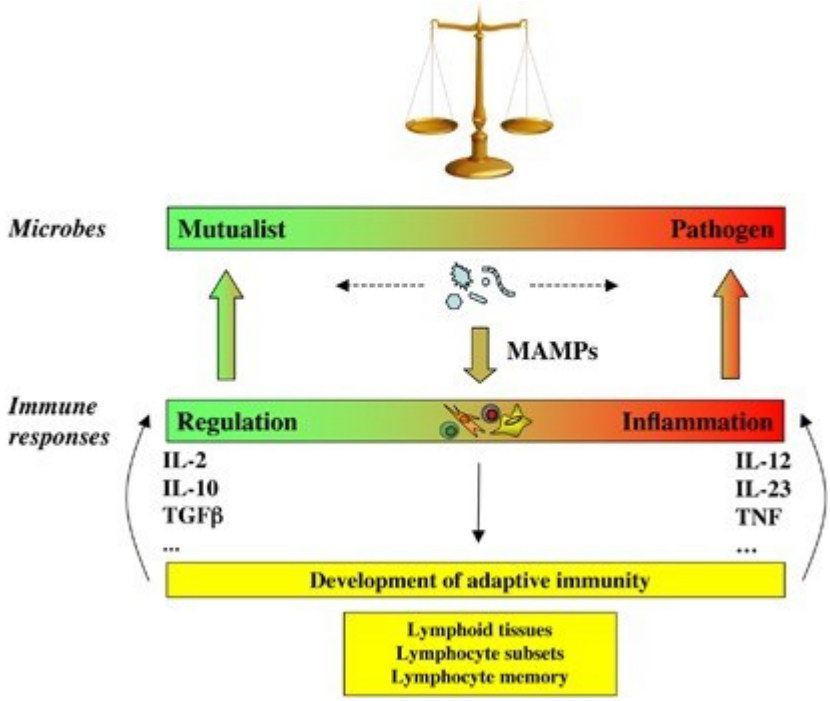


Figure 4. The continuum of dynamic equilibrium between microbial states and immune responses (Eberl, 2010).
MMAMPs: microbe-associated molecular patterns, IL-2: interleukin 2, IL-10: interleukin 10, IL-12: interleukin 12, IL-23: interleukin 23, TGFβ: transforming growth factor β, TNF: tumour necrosis factor.

1.3.1 Post-Weaning Diarrhoea in Swine Species

In intensive pig farming, the weaning is a critical moment in the life cycle of piglets. It is source of tremendous stressors since they are forced to adapt to nutritional, physical and immunological changes (Lynegaard et al., 2022). During the weaning, piglets are separated from the sow, fed with different diet, and animals need to adapt to new environment and litter mixing. All these changes may affect the inflammatory and immune status, predisposing animals to develop gastrointestinal disorders such as post-weaning diarrhoea (PWD). PWD has been reported as one of the most impacting diseases in swine farms globally and is source of huge losses to farmers trough increased morbidity, mortality, lowering animal performance resulting in increased costs of treatments and lower incomes (Rhouma et al., 2017).

Even if many pathogens could be involved as door openers in gastrointestinal disorders, *Escherichia coli* is the most abundant aerobic coliform bacteria in the colon. Among *E. coli* strains, there are some enteric pathogens expressing virulence factors causing hypersecretory diarrhoea in the host. Among them, some strains can produce enterotoxins that impair the structure and functioning of enterocytes disrupting the intestinal barrier. Exotoxins form *E. coli* have been grouped in four types: heat labile toxins (LT), heat stable toxins (STI and STII), and verocytotoxin type 2 variant (also known as Shiga-like toxins) that are involved in oedema disease. Another important

factor for bacterial colonization is the presence of adhesive fimbriae, whose are required for the mucosal binding thus allowing the replication and exotoxins production. Pathogenic strains of *E. coli* of swine species are mostly characterized by the presence of F4 and F18 fimbriae that are frequently observed in O138, O139 and O141 serotypes (Rossi et al., 2013).

PWD is mostly caused by enterotoxigenic (ETEC) and/or verotoxigenic (VTEC) *E. coli* strains which may trigger watery diarrhoea, dehydration, sudden deaths, and retarded growth (Lauridsen et al., 2017). Although the presence of pathogenic *E. coli* is widely implicated in PWD, the disorder is defined as multifactorial resulting from the interaction of the sow, piglets, environment, bacteria and farm management (Figure 5) (Rhouma et al., 2017). Influence factors of PWD occurrence can be classified in predisposing, contributing and determining causes. Genetic, immunity, weight and weaning age can be included into predisposing factors. Several studies demonstrated that a lower weaning weight and a short lactation period can increase the occurrence of PWD (Bogere et al., 2019). The colonization of β-haemolytic ETEC, expressing F4 of F18, of intestinal mucosa is strictly related to the interaction between fimbriae and receptors that allows pathogenic strains to adhere and produce exotoxins causing more severe infections (Figure 6) (Rossi et al., 2014). The presence of F4 and F18 fimbriae receptor is pivotal for the gut colonization from pathogenic strains of *E. coli*. The expression of intestinal receptor is genetically controlled and it determines the animal susceptibility to PWD (Luise et al., 2019). Contributing factor can be summarized including gut dysbiosis, litter size, sow parity, hygiene, feeding regimes, birth order and genotype (Bogere et al., 2019).

In this scenario, limiting the spread of PWD to raise the animal welfare and farm profitability is needed. On the other hand, to decrease the utilization of antibiotics, conventionally prescribed to control PWD, therefore alternative approaches are required.

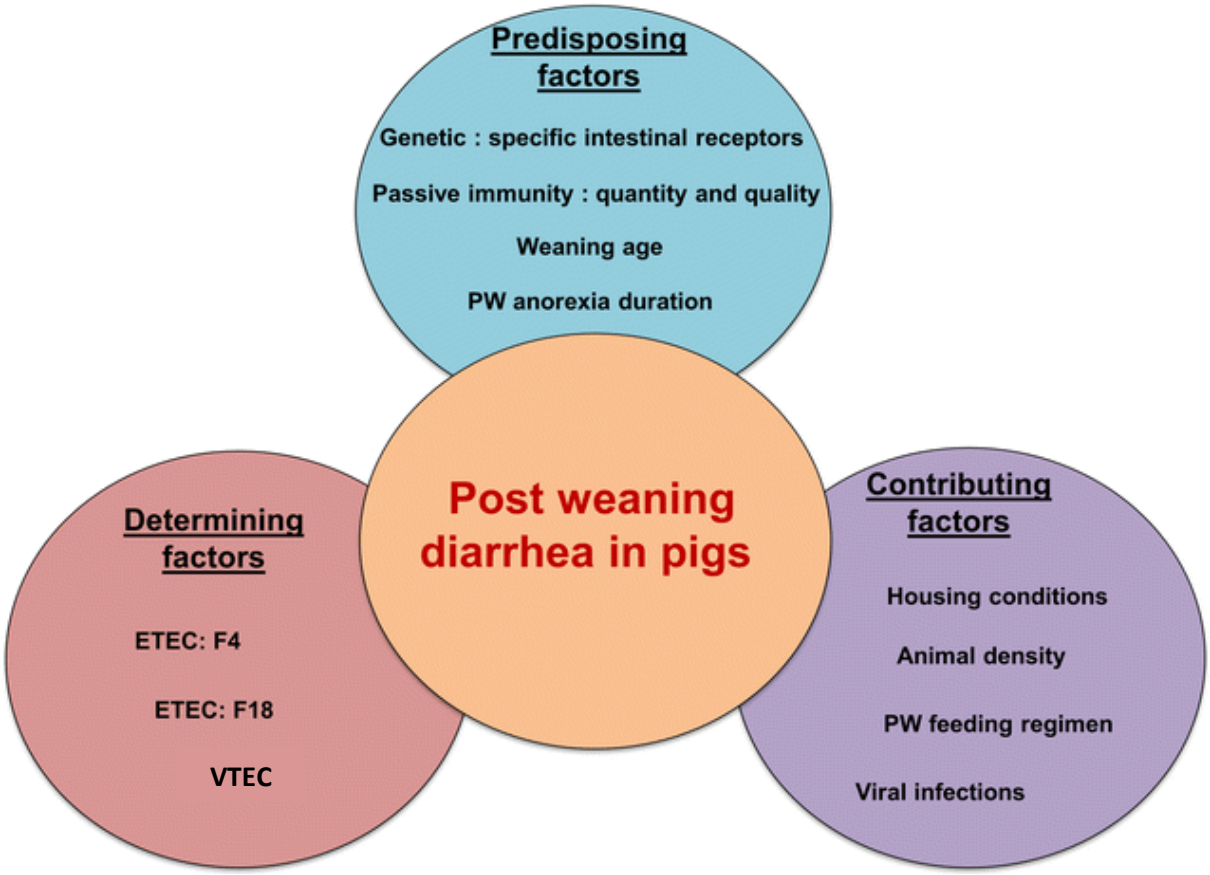


Figure 5. PWD is a multifactorial disease involving the interaction between predisposing, contributing and determining factors (Rhouma et al., 2017).
PW: Post weaning, ETEC: enterotoxigenic *Escherichia coli*, VTEC: verotoxigenic *Escherichia coli*.

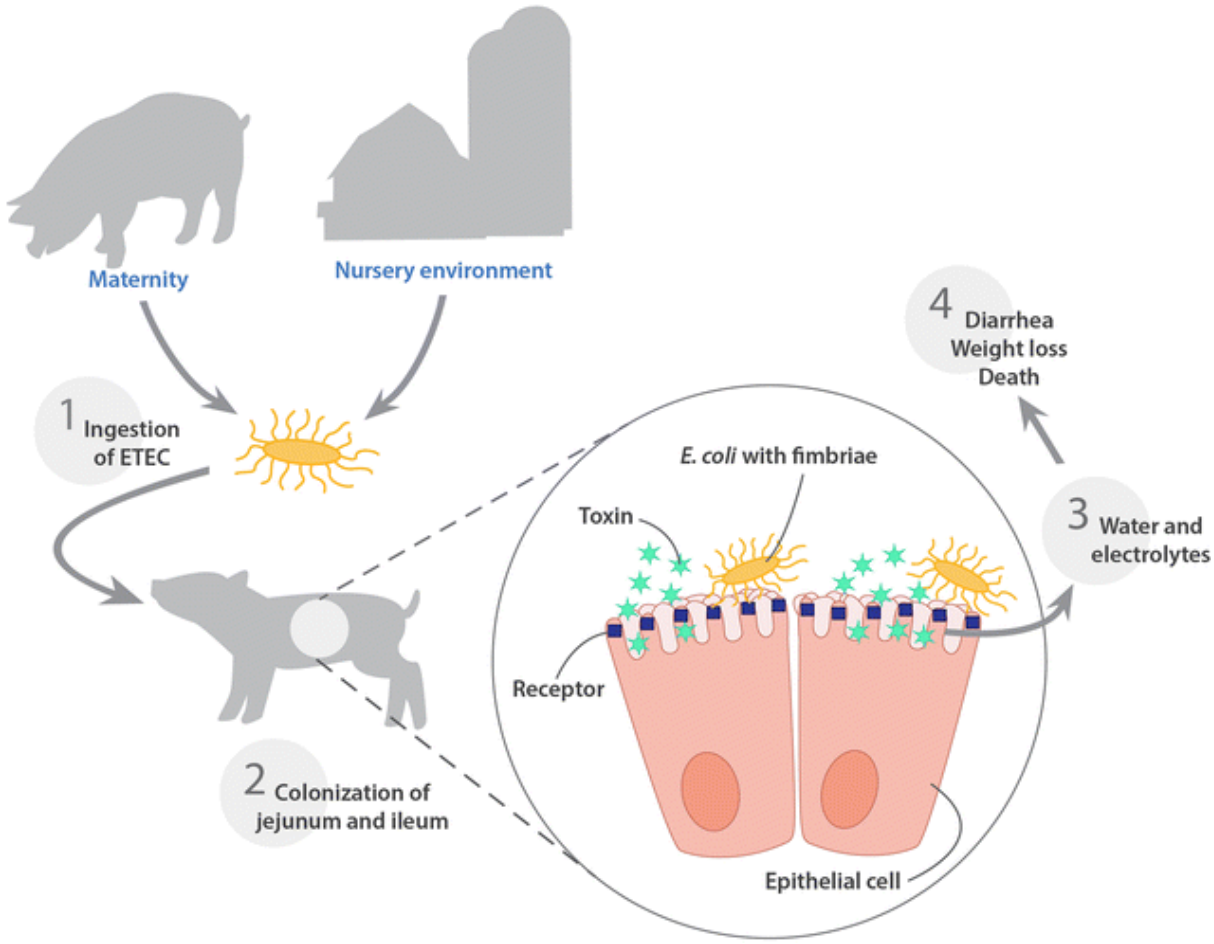


Figure 6. Scheme of principal steps involved in the development of post-weaning diarrhoea in pigs (Rhouma et al., 2017).

1.3.2 Neonatal Calf Diarrhoea

The neonatal calf's diarrhoea (NCD), also known as calf scours or enteritis, is a gastrointestinal multifactorial disease cause of one of the most important worldwide economic losses in dairy farms. The harmful impact of NCD can be fatal for young animals as consequence of hypovolemia and acidosis which can lead to anorexia and ataxia. In addition, the demanding of antimicrobial treatments and productivity losses also due to detrimental effect on further lactation performance increases the severity of this pathology (Prieto et al., 2022). NCD can be the reason of 75% of calf mortality during the pre-weaning period (Khawaskar et al., 2021). Moreover, NCD is also related to indirect losses due to higher morbidity, increased needs and costs of treatments and growth retardation, also with negative effects on further dairy career duration and productivity (e.g. delayed first calving) (Brunauer et al., 2021). NCD is a multifactorial disease where infectious agents, such as Rotavirus, Coronavirus, *Escherichia coli*, *Salmonella* spp., *Clostridium perfrigens*, *Cryptosporidium parvum*, and animal (age, immunity, general health status) and environmental-related factors (colostral

consumption, calf housing, hygienic conditions) can concur to its onset and incidence (Cho and Yoon, 2014; Windeyer et al., 2014).

Non-infectious causes are comprehensive of nutritional status, while infectious causes include a wide range of pathogen viruses, protozoa, and bacteria where one or more can be involved in the pathology development (Maier et al., 2022). Standard treatments for affected calves are oral or intravenous fluid therapy, isolation of infected and antimicrobial use (Smith and Berchtold, 2014). The use of antimicrobial treatments for NCD is still debated since antimicrobial resistance is currently an exponential threat for humans and animals. However, there is the risk for calves affected by diarrhoea for the development of septicaemia that require to receive antibiotics thus veterinary treatments are still needed (Smith, 2015).

Recent surveys data from Austria, Belgium, Portugal, and Scotland revealed that 52.5% of farmers and veterinarians used antimicrobials to treat calves' diarrhoea (Eibl et al., 2021). Methods recommended for the prevention of NCD include proper cow nutrition during pregnancy, dystocia management, reduction of environmental stress and contamination, and ensuring transfer of passive immunity to the calf via colostrum, enhancing the presence of specific antibodies in the gut lumen during the calf's most vulnerable age for NCD, and bolstering the calf's immunity via systemic uptake of antibodies (Al-Alo et al., 2018). Colostrum quality depends only partly on vaccination of the cow during pregnancy (Geletu et al., 2021; Menichetti et al., 2021). In calves, the microbial colonization and development of gut microbiota begins after birth, starting from maternal sources (Whon et al., 2021). Generally colonization process opens with facultative anaerobes with *Proteobacteria* species that consume oxygen in intestinal environment facilitating the further colonization of strict anaerobes (Koren et al., 2012; Shin et al., 2015). Gut inflammation followed by dysbiosis raises the risk of diarrhoea development and impairment of colonization pattern is related to higher frequency of gut disorders in neonates (Morrow et al., 2013). In light of this, the interaction between the environment and antibiotic treatments lead to an incomplete establishment of microbial ecosystem with further dysbiosis-induced diarrhoea in calves (Whon et al., 2021). Besides the ban of antibiotics as growth promoters in animal feed (Regulation EC 1831/2003), the persistent emergence of this problem prompted the European Union to introduce more limitations for veterinary drugs use (Regulation EU 6/2019). A specific focus was paid to the Highest Priority Critically Important Antimicrobials (HPCIA) (EMA et al., 2019; WHO, 2019). Therefore, a judicious use of antibiotic drugs is necessary in animal farming. In light of this, alternative strategies to reduce the incidence of NCD in calves are required.

1.4 Functional Nutrition

Nowadays the aim of nutrition is no longer to simply satisfy requirements; it also plays a pivotal role in the health and welfare of humans and animals (Domínguez Díaz et al., 2020). Functional feed ingredients and additives are capable to sustain the health status and decrease the pathologies incidence, and they thus became crucial as alternative strategy to replace or reduce antimicrobials in livestock (Lallès and Montoya, 2021).

Modern concept of food and feed is meant to provide products with an added value delivering more that the necessary nutrients and the satisfaction of hunger. Functional foods, feed, and nutraceuticals additives have been designed to increase health status and prevent several nutrition-related diseases.

The term "functional food" was used in Japan in 1984 to describe a food product able to increase human health (Domínguez Díaz et al., 2020). An important characteristic of functional food or feed is that they do not solely provide essential nutrients to satisfy requirements but they are composed of natural bioactive compound that provided at right doses may positively affect the welfare and life quality reducing risk of pathologies without exerting a particular therapeutic function (Doyon and Labrecque, 2008). Nutraceuticals were introduced by Stephen DeFelice and derived from terms "nutrition" and "pharmaceutics", defining them as food (or part of a food) which provides medical or health benefits. In the last two years, the outbreak of COVID-19 pandemic has lead to an increased demand of nutraceutical products around the world and it is estimated that their market size will overtake 990 billion by 2030 (Tang, 2022).

The investigation on alternative feeding strategies to increase intestinal health and resistance to develop gut disorders has become fundamental for the animal-derived food sector (Suresh et al., 2018; Lo Verso et al., 2020; Borrelli et al., 2021). Functional additives are not necessary designed for supplying nutrients; however, they are composed by bioactive molecules with beneficial effects on health. The use of functional additives in animal diet has gained an important attention due to their ability to support animal health, welfare and performance reducing the use of medicines. Several functional additives have shown that can positively impact the immune system, host metabolism, inflammatory pathways and antioxidant defence and modulating gut microbiota also with antimicrobial effects (Lopreiato et al., 2020; Majumder et al., 2020).

Among all the activities of functional ingredients and additives, antioxidant and antimicrobial activities are considered two pillars that elicit an important interest in research.

1.4.1 Antioxidant activity

One of the most important activities which characterizes functional compounds is the antioxidant effect which is defined as the ability of a substance to inhibit the oxidation (Granato et al., 2018). This activity supports the oxidative balance, and several classes of antioxidants can be found in natural extracts. Various phenolic compounds derived from secondary metabolism of plants, protect the organism from oxidation and they are regarded as natural antioxidants. They can be divided into five categories based on their function: free-radical scavengers, scavengers of non-radical oxidizing molecules, and compounds that stimulate the endogenous production of antioxidants. Antioxidants can be water soluble (polar) or liposoluble (apolar). They can be produced by endogenous metabolism, or they can be supplemented with the diet. The mechanisms of action of antioxidants have been categorized in three main types: hydrogen atom transfer (HAT), single electron transfer (SET) and transition metal chelation ability (Brewer, 2011).

Oxygen is a highly reactive nonmetal and oxidizing agent towards several elements and other compounds. Molecular oxygen, in the ground state, has two unpaired electrons with parallel spins in two separate anti-bonding orbitals (Gulcin, 2020). Due to this property, oxygen can accept two electrons from an electron donor. Redox reactions are crucial in biological systems, beginning from the synthesis of adenosine triphosphate (ATP) which is operated by a chain which uses O_2 as ultimate electron acceptor (Gülcin, 2012). In addition, O_2 is widely used in aerobic organisms for reduction and enzymatic processes (Elmastas et al., 2018). When the electron transfer becomes uncoupled, the process generates free radicals. A free radical is defined as chemical species capable of independent existence, possessing one or more unpaired electrons. This property makes them highly unstable compounds. They are internally produced as a normal part of metabolism within the mitochondria, through xanthine oxidase, peroxisomes, phagocytosis, inflammation processes, ischemia, arachidonate pathways, and physical exercise (Gulcin, 2020). Radicals essentially derive from three chemical elements: nitrogen, sulphur and oxygen. Free radicals derived from oxygen are defined as reactive oxygen species (ROS). Some of them have a half-life of few seconds (hydroxyl HO· half-life = 10^{-9} s) and they rapidly attack compounds in the surroundings. Probably, this damage is unavoidable and only the repairment process can be effective. Meanwhile other ROS are nonradical (singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2, hypochlorous acid HOCl) although they can generate other free radicals (Gulcin, 2020). ROS are constantly produced by aerobic organisms during normal cellular metabolism, respiration, autoxidation of xenobiotics or stressful conditions and they can disrupt all biomolecules including carbohydrates, proteins,

lipids and nucleic acids (Cakmak and Gülçin, 2019). ROS formation plays a key role in the maintenance of cell homeostasis by a system of antioxidant defence which allows them to maintain the correct balance. When balance is impaired, the oxidative stress downregulates several cellular functions exposing the host to tissue injury, accelerated cell death and development of pathologies (Sindhi et al., 2013; Apak et al., 2016). When the balance between prooxidants and antioxidants is shifted towards the prooxidants or when levels of antioxidants is low the state is called oxidative stress. This condition has been described as imbalance between production of ROS and organism ability to scavenge the reactive intermediates or to repair the damage (Kalin et al., 2015). The oxidative stress is often due to a reduction in antioxidant enzymes and the exogenous intake, contemporarily the number of reactive species derived from phagocytes is raised during chronic inflammatory states (Gülcin, 2012). Oxidative stress is involved in the development of several diseases and is known as predisposing factor.

Antioxidants can be found in microorganisms, animals and plants secondary metabolites or they can be synthesized by chemically in laboratories for cosmetics. In general, plants are a rich source of natural antioxidants. However also algae, fish/shellfish and marine bacteria have been considered as source of antioxidant molecules. One of the most cost-effective opportunities for animal nutrition are by-products from food and agriculture sectors that can be exploited for supplementing functional compounds in animals' diets (Correddu et al., 2020).

The strength of antioxidants is influenced by their structural properties that determine their intrinsic reactivity to hence the antioxidant activity towards radicals and reactive oxygen species. Their efficiency also depends on the location and concentration in the organism and the kinetics of the reaction determines their short- or long-term protection taking into account the thermodynamics of the reaction, the rate and the extent of reaction towards a specific oxidant (Shahidi and Zhong, 2015).

Antioxidant therapy renewed its interest in many studies also related to cancer research (Salehi et al., 2018; Ferdous and Yusof, 2021). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), α-tocopherol, carotenoids and vitamin C are enzymatic and non-enzymatic antioxidant systems present in the body which regulate the oxidative balance (Kalin et al., 2015). Exogenous antioxidants that comes from the diet are mostly present in plants and their impact has been demonstrated as positive decreasing the risk of development of several pathologies and aging process (Kumar et al., 2016). Dietary antioxidants are a wide group of molecules with different bioactive effects, their consumption through their mixing can also act synergistically when combining separate antioxidant molecules from different sources (Frazzini et al., 2022).

Antioxidants can be essentially classified as direct or indirect depending on their mechanism of action. Direct antioxidants have a short half-life and they require to be supplemented frequently at relatively high dosage to maintain their efficacy. On the other hand, indirect antioxidants increase the antioxidant capacity of cells triggering specific genes encoding for antioxidant proteins, showing longer activity compared to the direct ones. In particular, they upregulate the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which regulates the antioxidant response in the host (Lee et al., 2017). Nrf2 is a transcription factor that activates the phase II detoxifying and cis-antioxidants response genes such as oxidoreductase 1 (NQO1), glutathione S-transferase (GST), heme oxigenase-1 (HO-1), glutathione peroxidase (GSH-Px), glutamatecysteine ligase (GCL), CAT, SOD, uridine diphosphateglucuronosyltransferase, glucuronosyltransferase, and the thioredoxin/peroxiredoxin system (Baird and Dinkova-Kostova, 2011). Several studies demonstrated the efficacy of dietary antioxidants from natural sources in activating Nrf2 inducing the expression of detoxifying enzymes (Sahin et al., 2010; Saw et al., 2012; Lee et al., 2017).
The dietary supplementation of bioactive compounds with antioxidant capacity is considered important for functional nutrition in order to combat oxidative stress to prevent pathologies and decrease antibiotic treatments.

1.4.2 Antimicrobial effect

Another important feature of many functional feed ingredients and additives is the antimicrobial effect that is intended as a wide complex of different mechanisms able to limit the bacterial, viral, fungi, protozoa growth, replication or causing the microorganisms death, which result of particular interest against pathogenic strains.
Focusing on the antimicrobial activity towards bacteria, bacterial cell wall is mainly composed by peptidoglycan made of repeated units of N-acetylmuramic acid and N-acetlyglucosamine cross-linked by small molecular weight amino acids that are pivotal for the bacterial cell protection (Humann and Lenz, 2009). Plant-derived compounds have proved interesting effect in controlling the synthesis of bacterial cell wall (Upadhyay et al., 2014). Some compounds may interact with protein attached to bacterial cell walls enhancing the membrane permeability thus impairing their integrity. Phenolic compounds (flavonoids, flavones and flavonols) revealed cell wall lysis abilities due to their capacity to dissolve surface proteins through the creation of complexes with bacterial cell walls (AlSheikh et al., 2020). Polyphenols from tannins impact the lipophilic cell layers. Alkaloids are antimicrobial through intercalating the DNA and cell wall of microorganisms. Selim et al. (2012) observed that flavonoids can

frame pores in the cell wall, harming the bacterial survival. Mammalian cells are of zwitterionic nature and do not have affinity with the positively charged antimicrobial peptides, rendering them selectively toxic toward bacteria (Le et al., 2017). Aside from membrane activity, intracellular targets are increasingly being investigated. Even if some mechanisms of action are not completely known, the ability to interfere with the organization of cellular membrane leads to a reduced membrane potential and inhibits the APT synthesis resulting in bacterial death. As example, cinnamaldehyde inhibits the glucose uptake and impair the ATPase-dependent metabolism depleting glucose provoking cellular death (Negi, 2012; AlSheikh et al., 2020).

Some natural compounds can interact with antibiotics reinstating the efficiency and the susceptibility in multidrug resistant bacteria (Lorenzi et al., 2009) through restricting the activity of enzymes responsible to resistance (β-lactamases, penicillases) and inhibiting the efflux pumps activity. Other studies demonstrated some synergisms among plant extracts and antibiotics (Rodrigues et al., 2009; Chan et al., 2011).

In addition, bioactive compounds can exert antimicrobial activity by limiting the biofilm formation, a matrix of exopolysaccharides integrated with microbial population. The anti-biofilm activity can be elicited by modifying the gene expression and the quorum sensing signalling (autoinducer-2-dependent quorum sensing) (Amalaradjou et al., 2011; Yim et al., 2011; Upadhyay et al., 2012). Quorum sensing is a mechanism that allow a cell-to-cell communication in microorganisms, that bacteria can use to induce the biofilm formation as protection tool under unfavourable conditions such as antibiotics presence. The inhibition of this signalling leads to inhibited biofilm production exposing bacterial surface to antimicrobial substances.

Other important mechanisms are related to the control of the expression of bacterial virulence factors such as capsule polysaccharides. The presence of capsule protect microorganism to phagocytosis and supporting them in the biofilm formation (Hyams et al., 2010). Natural compounds such as sodium salicylate, bismuth subsalicylate have shown to inhibit the capsule formation by regulating the gene expression of this virulence factor. Additionally, several pathogens adhere to enterocytes using the receptor-biding mechanism towards the production of surface proteins whose production can be inhibited by phytochemicals (Shah, 2003; Walencka et al., 2007). Exploiting the variety of the antimicrobial effects of functional feed additives has become fundamental to use them as therapeutics to combat the raising issue of antibiotic resistance.

1.4.3 Gut microbiota colonization in young animals

The entire gut microbiota has different roles in the host, contributing to fundamental activities such as digestion, vitamins production, intestinal villi functionality, immune response and protection from pathogens. Gut microbiota is a high-complexity ecosystem with dynamic composition and diversity that undergoes to important changes over time and along the gastrointestinal tract (Gresse et al., 2017). The early microbial colonization in piglets begins after birth by the consumption of colostrum and milk from the sow, which promotes the spreading of lactic acid bacteria for establishing a milk-oriented microbiome (Frese et al., 2015). Different strains of *Escherichia coli* and *Streptococcus* spp. contribute to create an anaerobic environment favouring the colonization by other strictly anaerobic genera such as *Bacteroidetes, Lactobacillus, Bifidobacterium* and *Clostridium* (Petri et al., 2010). The use of nursing mother or animal genetics importantly impact the development of gut microbial community, and the suckling period offers an important window for microbiota modifications. After weaning, piglets are fed with cereal-based diet and high concentration of proteins (≈20%) even if modern formulation techniques focus more on the aminoacidic profile thus decreasing the percentage of total crude proteins. Due to high protein levels, several studies reported that during the weaning period *Lactobacillus* genera tend to decrease and the microbial diversity reduces. At the same time, *Clostridium* spp., *Prevotella* spp. or facultative anaerobes form Proteobacteriaceae are positively impacted (including *E. coli*) (Bian et al., 2016).

Regarding the bovine species, gut microbial environment in calves is reported to increase in complexity and diversity with the growth, as result of age and dietary modifications. First bacterial genera in the colonization detected in calf meconium are *Citrobacter*, *Lactococcus*, *Leuconostoc,* and *Lactobacillus (*Mayer et al., 2012). After 24 hours the diversity between microbial composition of meconium and faecal samples due to an increased diversity from an establishment of a complex microbiome very early in life. The abundance of *Bifidobacterium, Lactobacillus, Fecalibacterium,* and *Enterococcus* genera drops with calves growth (Malmuthuge, 2017). Mayer et al. (2012) highlighted that faecal microbial composition in twin calves was more similar compared to siblings indicating that genetics can drive the individual gut microbiota composition. *Firmicutes* and *Bacteroidetes* are reported to be predominant during the first three weeks of life and the relative abundance of this phyla is widely different between the tissue and content of gut regions (Foditsch et al., 2015). Studies on gut microbiota in ruminants highlighted how newborn calves are colonized by facultative anaerobes that contribute to create anaerobic environment suitable for the colonization by strictly anaerobic bacteria such as *Lactobacillus* and *Bacteroides* and the modification of microbial environment is age dependent (Arshad et al., 2021). The colonization of

gastrointestinal tract also depends on the environment that newborns are exposed. In general, the intestinal microbiome of neonatal calves showed a limited number of phyla (Protebacteria, Firmicutes, Actinobacteria and Bacteroidetes), indicating a shared microbiota between meconium and vaginal vestibulum of mothers (Wang et al., 2019a).

1.4.4 Gut microbiota modulation

The microbiota is considered as the assemblage of microorganisms present in a defined environment. The microbial census can be established using molecular methods relying on the analysis of 16S rRNA, 18S rRNA genes or other genomic regions, amplified and sequenced form biological samples.

Bacterial cells present in the mammalian gut are more numerous than other eukaryotic cells in the entire body, therefore they significantly impact the host's health. The modulation of intestinal health through nutritional approach has acquired an important value. The use of the term "gut health" has been used frequently in the last years, however a precise scientific definition of it is still lacking. The concept of intestinal health is a complex dynamic and relative and the absolute state of healthy gut is probably not possible to be defined. The prevention of multifactorial disease, immunomodulation, utilization of nutrients and growth performance of animals is supported by a healthy intestinal environment (Heo et al., 2013; Pieper et al., 2016).

Three important complex systems influence each other to determine the individual dietary response: diet, microbiota, host physiology and metabolism. These systems are strongly connected and interdependent (Kolodziejczyk et al., 2019).

Feed is one of the most important factors that connects the body organs with external environment through the gastrointestinal tract (GIT) that can be used to modulate gut microbiota (Yadav and Jha, 2019). Intestinal microbiota is strictly linked with the host, diet and itself. Commensal bacteria play a key role for the host's health and metabolism, meanwhile the pathogenic strains can cause harmful effects. Therefore, influencing the gut microbiota by nutrition, in order to maintain a correct balance (defined as eubiosis), is fundamental. Bacterial communities can be subdivided into three main types: dominant ($> 10^6$ CFU/g sample), subdominant (from 10^3 to 10^6 CFU/g sample) and residual bacteria ($< 10^3$ CFU/g sample) (Aland and Madec, 2009).

The beneficial intestinal microbiota has a protective role of defence towards pathogenic bacterial strains and contributing to host metabolism and epithelial integrity.

Diet is crucial for modulating the microbial balance, and nutritional approaches should consider all interactions among bacteria, gut epithelium and environment

(Yadav and Jha, 2019). In light of this, the supplementation of different feed additives has been investigated in last years. As example, probiotics (viable microorganisms) or prebiotics (undigestible oligosaccharides) may affect the gut microbiota of the host influencing intestinal health (Heinritz et al., 2013).

The intestine hosts the highest number of immune cells and mucosa-associated microbial population directly interact with these cell types (Belkaid and Hand, 2014; Mowat and Agace, 2014). Mucosal microbiota is the first line of defence against pathogens by competitive exclusion and immune modulation (Ma et al., 2018). The secretion of mucosal immunoglobulin, such as immunoglobulin A (IgA), modulates the colonization of gut microorganisms. The symbiotic role of balanced microbiota is important to metabolise some kind of toxins, synthetise vitamins, support the maturation of enterocytes, immune system and barrier function (Yang et al., 2016; De Vries and Smidt, 2020).

The early establishment of gut microbiota is important for the maturation of barrier function and immune system affecting animal health and growth. Shanahan et al. (2017) showed that microbiota is linked to the sensitivity to growth hormone, therefore gut microbial environment can enhance the growth of neonates. In addition, the early establishment of gut microbiota has been demonstrated to develop a long-lasting effect along the animal life (Everaert et al., 2017; Schokker et al., 2018). The luminal microbiota is distinctly different from the mucosa associated one, also with different role and interactions. Luminal bacteria are more liked to digestion and secretion of metabolites, whereas the mucosa-associated microbial population is directly associated with immune cells of the host by crosstalk communication (Duarte and Kim, 2022). The mucosa-associated microbiota can colonize by binding mucin glycan on epithelium surface and is more susceptible to dietary changes (Etienne-Mesmin et al., 2019). GIT is composed by different segments and niches (lumen and mucosa) that are not completely independent. Microorganisms can co-inhabit mucosa an intestinal lumen. Therefore, gut microbiota can be influenced by same factors reposing in different ways (Galley et al., 2014; Zhang et al., 2018).

Most of genes that influence the mucosal microbiota are linked to the immune response, hypothesising that the immune system applies a selective pressure to promote favourable communities (Honda and Littman, 2016).

Gut epithelium is composed of enterocytes, gut-associated lymphoid tissue (GALT), and the mucus layer. Bacterial population associated with the mucosa, engage a complex crosstalk with epithelial cells that develop a dynamic interaction critically important for the nutritional and immune status of GIT (Ma et al., 2018). In particular, M, dendritic and epithelial cells recognize antigens in the intestinal environment inducing then the expression of Toll-like receptors (TLR), nucleotide-biding

oligomerization domain-like receptors that trigger the recruitment of T and B lymphocytes (Gutzeit et al., 2014). Presence of T and B cells, along with cytokines, chemokines and microbial metabolites play a pivotal role in the maintaining of intestinal homeostasis and the inflammatory status. Therefore, understanding the roles of intestinal microbiota and their interaction with the diet is essential in feed formulation and dietary supplementation in animal livestock (Duarte and Kim, 2022).

1.5 Active ingredients and additives as alternatives to antibiotics

During the neonatal and weaning periods, in order to support animal health and performance antibiotics have been traditionally included in animal feed. However due to the raising issue of AMR the European Union introduced the full ban for in-feed antibiotic usage as growth promoters from January 2006. Despite the technological development in modern medicine, the hopefulness of antibiotics is faded away by the appearance of different resistance mechanisms that caused important concern against the frontline antibiotics. The drugs production from the late 1960 is critically dropped and new medications require long periods of time for testing and be accepted by authorities (AlSheikh et al., 2020).

There is a growing need for finding valuable alternatives to antibiotic treatments, meant as any substance capable to reduce the use of antibiotics, to prevent or treat microbial infections.

Each substance which may be able to reduce the use of antibiotic drugs can be defined as alternative to antibiotics. Among these, functional feed additives possess beneficial compounds that may improve performance, health and welfare of animals reducing the incidence and severity of intestinal disorders. In this field, natural extracts and phytochemicals renewed their interest due to their content of bioactive molecules with functional properties. Dietary supplementation of tannins, coumarins, flavonoids, essential oils, alkaloids, organic acids do not normally induce resistance (Lewis and Ausubel, 2006). Phytochemicals have shown in many studies several mechanisms against bacterial cell: including the increase of cell permeability, leakage of cellular constituents, alteration of cell wall, ATP depletion, inhibition of protein synthesis, modification of pH, intracytoplasmic alteration, DNA damage and inhibition of quorum sensing mechanisms causing bacterial inhibition or death (El Kolli et al., 2016; Gemeda et al., 2018; Farha et al., 2020). Animal diets are continuously adjusted to optimize the animal health and growth controlling the feed costs. Feed supplementation could include prebiotics, probiotics, organic acids and phytochemicals. Probiotics can directly modulate the gut microbiota with competitive exclusion and stimulating the secretion of health-promoting metabolites. Prebiotics are

indigestible oligosaccharides and fibres that can shape microbial population by suppling the substrate for their growth. Organic acids decrease the pH limiting feed spoilage and lowering the survival of pathogens in the gut. In addition, some short chain fatty acid nourish the gut mucosa improving its morphology and functionality (Allen et al., 2013).

Numerous commercial products are available on the market. However, the true efficacy of many additives is still unclear due to inconsistent experimental results. Observed disparities may include the experimental conditions, animal age, genetics and health status. For these reasons is important to investigate their effectiveness. Even if several feed additives have been studied as alternatives to antibiotics, observed results are still contradictory. However, due to the needing to decrease antibiotics use, the supplementation of functional compounds may be beneficial when fed to animals (Liu et al., 2018). For these reasons, is still fundamental to better understand the functional effects of feed additives to develop novel dietary approaches as alternatives to antibiotics to cope with the increasing problem of AMR. In the subsequent chapters are described the products (algae, phytochemicals, organic acids, and microbial products) evaluated as alternatives of antibiotics object of studies and papers collected in the following PhD thesis.

1.5.1 Algae

The European Union has recently adopted a strategy for developing the bioeconomy and algae represents an emerging biological resource of great importance for its potential application in several fields.

Algae are a heterogeneous group of autotrophic, photosynthetic, eukaryotic, thalloid and largely aquatic organisms, ranging from unicellular microalgae to complex giant seaweeds. Macroalgae, also called seaweeds, are macroscopic, multicellular aquatic plant-like organisms, which can be divided into three main groups: brown, red and green algae. Two of the most studied species of brown algae for animal nutrition are *Ascophyllum nodosum* and *Laminaria sp*. According to Evans and Critchley (2014) prebiotic potential of *A. nodosum* has shown to be five times higher than inulin (used as reference) with additional performance-enhancing benefits in pigs' diet. The content of glutamic acid in *A. nodosum* showed to decrease the dry matter (DM) intake in calves (Erickson et al., 2012). Another interesting species for feed formulation is *Laminaria digitata*. Different authors demonstrated that *Laminaria* spp. extract (rich in laminarin and fucoidan) improved weight gain, feed intake and reduced faecal pathogens abundance in pigs (Gahan et al., 2009; McDonnell et al., 2010).

Seaweeds are rich in bioactive compounds, which may improve animal's health. Polyphenols are a group of secondary metabolites of plants, ranging from simple molecules, such as phenols, to complex ones, such as tannins, phlorotannins and flavonoids. The main properties of flavonoids are antioxidants, antibacterial, anticancer and antiviral activities (Mishra et al., 2013). Flavonoids are contained in substantial percentages in seaweeds, especially in red algae. Different studies confirmed that phlorotannins, mainly produced by brown algae, are powerful antioxidants showing the ability to inhibit matrix metalloproteinase and hyaluronidase, which are involved in the inflammatory process (Kim and Himaya, 2011). The natural content of phlorotannins in brown algae ranges from 1% to 14% of DM, with the highest content in *A. nodosum* species (14% of DM). Moreover, according to Choi et al. (2016), the brown alga *Ecklonia cava* displayed high content in fucoidans and phlorotannins, which showed beneficial effects on performance, digestibility and gut health in pigs.

Carotenoids are a class of pigments produced by algae and plants. They are mainly produced to protect the chlorophyll from photodamage by scavenging Reactive Oxygen Species (ROS) formed by light exposure. Thus, they turn out to be powerful antioxidants. Carotenoids in brown, red and also green algae are primarily formed by β-carotene that can be converted in vitamin A into the gut.

Microalgae are plant-like, single-celled organisms sized from 0.2 to 2.0 μm that can reproduce more rapidly than terrestrial plants by single cell division (Christaki et al., 2011). They are currently promoted as new source of valuable nutrients and bioactive compounds for human and animal consumption (Enzing et al., 2014). Microalgae presents advantages compared to terrestrial crops and they could represent a new source of protein to reduce the use of soybean, poly-unsaturated fatty acids, bioactive compounds with antioxidant and antimicrobial activities. Starting from the cultivation of microalgae, that is waste-less, ecologically pure and resource saving process and they can grow in a highly controlled or in open-culture systems such as lakes (Mata et al., 2010; Priyadarshani and Rath, 2012). They do not compete with terrestrial crops for soil, they can be grown unfavourable environment for crops: non-potable water, arid and marginal areas. Microalgae possess a more efficient photosynthesis reducing "greenhouse effect" producing more biomass and oxygen than terrestrial plants (Gouveia and Oliveira, 2009). Thousands of species of microalgae exists on the planet, most of them are edible and suitable for human and animal nutrition (Khan et al., 2018). Principal species of microalgae currently studied for animal nutrition are *Chlorella* spp., *Arthrospira* spp. (commonly known as Spirulina) and *Schizochytrium* spp. Even if cyanobacteria are often referred as "blue-green algae" traditionally included among the algae, prokaryotes are excluded from the definition of algae (Nabors and

Murray, 2004). However, cyanobacteria have similar characteristics to algae: behaviour, growing conditions and their traditional nomenclature (blue-green algae) (Madeira et al., 2017). The use of microalgae for animal feed started during the end of last century and it was related to their ability to enhance feed nutritional value due to their nutrients and functional compounds content (Khan et al., 2018).

1.5.2 Phytochemicals

Among the possible alternatives to antibiotics, phytochemicals play a very important role. They are secondary plant metabolites also known as phytobiotics, phytogenics or herbal and botanical compounds (Valenzuela-Grijalva et al., 2017). They are currently used as functional feed additives to replace antibiotics and also to increase animal performance due to their different antimicrobial mechanisms (Figure 7) (González-Ríos et al., 2016). Main bioactive compounds of phytochemicals are polyphenols, and their content or composition is largely variable according to the plant, anatomical part of the plant, geographical origin, harvesting season, environmental factors, storage conditions, and processing techniques (Gadde et al., 2017).

Recently, phytochemicals have been investigated as natural growth promoters in ruminants, swine, and poultry. Their complete plethora of mechanisms of action are not completely known. However, the beneficial effects of phytochemicals have been attributed to antimicrobial and antioxidant properties. In addition, the supplementation of natural extracts in feed formulations showed to modulate the gut microbiota, also inhibiting pathogens, that may enhance animal performance (Kim et al., 2015). One of the most important features of phytochemicals is the antioxidant effect that reduces oxidative stress promoting health (Settle et al., 2014). Phytochemicals showed to have immunomodulatory effect towards activation of immune cells proliferation, cytokine modulation, and raising antibodies level (Lillehoj et al., 2018). Some phytogenic additives can enhance the feed palatability, leading to higher feed intake (Bartoš et al., 2016). This may be a result of different activities such as increased antioxidant status of feed, decreased microbial colonization and stimulation of appetite (Velasco and Williams, 2011). Phytochemicals also demonstrated positive impact regarding intestinal function, such as digestive secretion and nutrient absorption. These effects have been attributed to higher secretion of saliva, enzymes and mucus, lowered bacterial load through antibacterial activity, prebiotic effect, improved gut morphology thanks to antioxidant and anti-inflammatory activities (Costa et al., 2013; Wall et al., 2014; Valenzuela-Grijalva et al., 2017).

With the increasing availability of several plant-based alternatives to antibiotics on the market claimed with several efficacies, we need to understand more regarding their mechanisms of action and their possible synergies offered by their combination. Using optimal combinations of these alternatives coupled with good management and husbandry practices will be pivotal for the future development of animal farming. More data are necessary to elucidate mechanisms of action, delivery methods, optimal concentrations, and possible combinations.

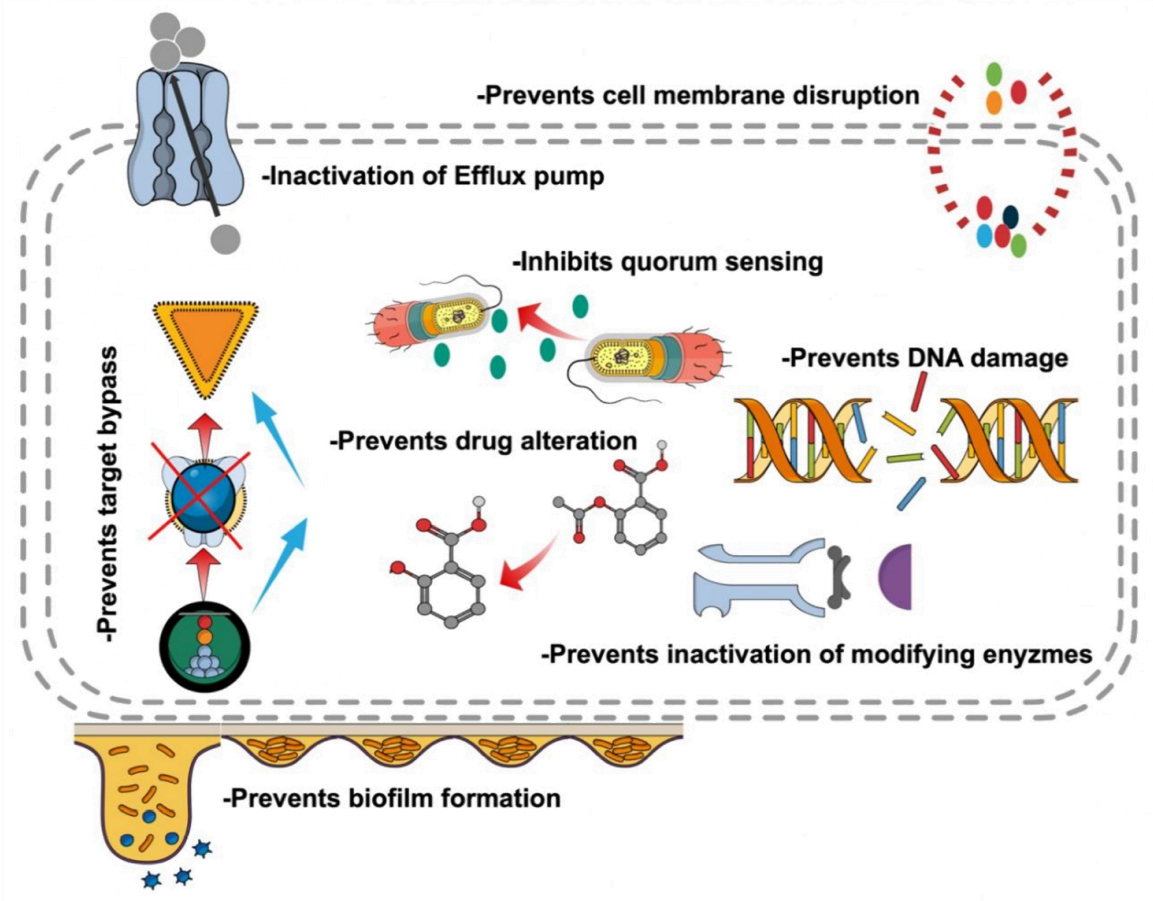


Figure 7. Principal mode of actions of phytochemicals and their effectiveness against bacteria (AlSheikh et al., 2020).

Tannins

Tannins can be found in several plant species. Some tannins-richest plants are the mimosa, chestnut, quebracho, maple tree, acacia, oak, and eucalyptus. Also fruits like blackberry, pomegranate, grapes, cranberry, and persimmon can be an interesting source of tannins. Tannins have a defensive role for plants to protect themselves from insects and predators due to their bitter taste (Farha et al., 2020). From a chemical point of view tannins are a class of polyphenols, secondary metabolites of plants, with a molecular weight ranging from 500 to 30,000 Da (Serrano et al., 2009). They can be

classified as hydrolysable (pyrogallol) or condensed tannins (proanthocyanidins). Hydrolysable tannins can be hydrolysed in monomers by chemical or enzymatic treatments. These are composed of phenolic acids and polyols (commonly glucose) grouped into gallotannins and ellagitannins (Khanbabaee and Van Ree, 2001). Condensed tannins (1-30 kDa) are more concentrated in plants compared to hydrolysable ones (500-3000 Da) (Ebrahimnejad et al., 2014). Tannins have a different behaviour in intestinal environment, the hydrolysable ones are degraded during digestive process whilst condensed tannins can not be absorbed by the gut (López-Andrés et al., 2013).

Tannins can inhibit the growth of several microorganisms showing both bactericidal and bacteriostatic effect depending on their concentration, class of tannins, and structural properties. As polyphenols, tannins are rich in phenolic groups that are responsible for their functional characteristics. In general, higher antibacterial effect has been observed with hydrolysable compared to condensed tannins (Caprarulo et al., 2020). This is probably due to the presence of galloyl groups that play an important role for antibacterial effect (Ekambaram et al., 2016; Farha et al., 2020). Siderophores that transport iron are essential for bacterial growth. Organic compound produced by bacteria are useful to bind and solubilize iron in the external environment for their availability. Tannins can chelate ferric iron leading to inhibition of bacterial growth. In addition, tannins can precipitate with iron forming a lattice (Engels et al., 2011; Bag et al., 2013). However, iron seems to be less important for probiotic bacteria survival. Some studies demonstrated that tannins did not influence the growth of lactic acid bacteria suggesting that they have stronger antibacterial properties against pathogens without negatively impacting on probiotic bacteria (Chan et al., 2016; Chan et al., 2018b; Chan et al., 2018a).

Another important antimicrobial property of tannins is the inhibition of cell wall synthesis by direct binding to peptidoglycan destroying the integrity of bacterial wall and increasing the susceptibility to osmotic lysis (Dong et al., 2018). In addition, tannins can affect the membrane potential increasing permeability of bacterial cell membranes leading to cell death. Some classes of tannins can interact with lipopolysaccharides of Gram-negative membrane. Proanthocyanidins have shown to bind with lipopolysaccharides impairing membrane integrity (Alshaibani et al., 2017). Tannins have also been shown to limit the bacterial biofilm formation. The binding ability on cell wall has been reported to reduce the produced biofilm (Kyaw et al., 2011). The inhibition of quorum sensing is also responsible for reduced biofilm production. This particular mechanism is used to control the gene expression and is based on secretion of particular inducer signals. Tannins can impair the signalling transfer thus negatively impacting the quorum sensing (Farha et al., 2020).

Tannins have shown to inhibit the expression and functionality of several enzymes that are produced as virulence factors such as protease, phospholipase, urease, neuraminidase, and collagenase (McCall et al., 2013; Quosdorf et al., 2017). Other studies demonstrated the inhibitory activity of tannins towards bacterial adhesion by influencing the surface hydrophobicity and cell aggregation activity (Gupta et al., 2012). Rasooly et al. (2019) reported that tannins can inhibit the production of staphylococcal toxins. Ellagitannins were shown to decrease the toxin production of multidrug-resistant *Clostridium difficile* (Sukumar and König, 2018).

Tannins are potent antioxidants that have been widely applied to food industry and medical fields. It is well known that condensed and hydrolysable tannins have higher antioxidant potential than simple phenols, since the number of hydroxyl groups and the polymerization degree are directly correlated with the free radical scavenging capacity (Ricci et al., 2016).

Anti-inflammatory properties of tannins are linked to the antioxidant capacity. Several *in vitro* studies demonstrated that tannins supplementation can reduce the pro-inflammatory mediator secretion and modulate the cytokine expression. Their activity may be more related to the radical scavenging ability than the inhibition of inflammatory cytokines production (Park et al., 2014; Liu et al., 2015a; Liu et al., 2015b). However, phlorotannins from brown alga *A. nodosum* displayed an inhibitory effect towards cytokine release decreasing the inflammatory status (Dutot et al., 2012). Considering their high content of bioactive compounds and beneficial properties widely reported in literature, tannins can be considered interesting functional feed additives for feed formula in livestock.

1.5.3 Organic acids

Organic acids are compounds with acidic properties, mostly consisting of carboxylic acids (-COOH). They can be divided into i) short chain fatty acids (SCFA) for organic acids with less than 6 carbon atoms; ii) medium chain fatty acids (MCFA) from 7 to 10 carbon atoms; and long-chain fatty acids (LCFA) with ≥11 atoms of carbon.

Organic acids were historically used for feed sanitization reducing fungal growth and preventing also bacterial growth. Some of SCFA such as acetate, propionate and butyrate are produced by the intestinal microbiota (Khan et al., 2022). Literature studies investigated their growth-promoting effects in monogastric animals. The observed results showed that organic acids may improve the feed utilization, reduce the microbial load and supporting intestinal health (Khan et al., 2016; Baghban-Kanani et al., 2019). Broilers infected with *Salmonella typhimurium* and *E. coli* that received a dietary supplementation of organic acids showed higher growth performance

compared to unsupplemented groups (Emami et al., 2017; Adhikari et al., 2020). Obtained results have been reported as the effect of nutrients digestibility enhancement, reduction of pathogens, reduced ammonia and other harmful metabolites production (Khan et al., 2016). Organic acids have shown to increase penetrate the bacterial cell membranes. After their penetration, protons are released by organic acids resulting in a reduction of intracellular pH. The process of pH lowering leads to an enzymatic reaction that forces bacterial cells to release protons and accumulate anions (Khan et al., 2022).

It has been reported that dietary fiber may be used as substrate to SCFA that nourish enterocytes and inhibit pathogens (Yudiarti et al., 2020; Scicutella et al., 2021).

The group of lactic, fumaric and citric acids are commonly used to decrease the pH of the stomach, creating an unfavourable environment for acid sensitive bacteria. Lowering the pH in cytoplasmic environment, bacterial transport systems and enzymatic reactions and energy production are disrupted (Scicutella, 2021). On the other hand, acetic, propionic, butyric and sorbic are also able to lower the pH, but additionally they can directly interact with bacterial cell wall (Papatsiros et al., 2014). In particular, through targeting the cell wall, membrane and metabolic pathways organic acids can impair the bacterial replication, protein synthesis, and function (Ricke, 2003; Dittoe et al., 2018).

Tributyrin

Tributyrin (butanoic acid 1,2,3-propanetriyl ester) is a single molecule composed by three butyric acids esterified with a glycerol molecule (Zhang et al., 2020). When hydrolysed by lipases, tributyrin releases three butyrate molecules resulting in a single compound three-fold more powerful than butyric acid. Tributyrin does not possess the strong odour of butyric acid thus not impairing the feed palatability. It is well known that butyric acid such as SCFA exerts positive effects to intestinal health nourishing enterocytes and inhibiting the bacterial growth. In particular, butyrate stimulates healthy cell proliferation, and inhibits cancer cells growth by increasing their apoptosis (Canani et al., 2012).

Butyric acid favourites the water absorption in the gut decreasing diarrhoea occurrence. In addition, it inhibits the pro-inflammatory transcription factor NF-kB, leading to lower levels of cytokines produced by intestinal cells (Liu, 2022). Previous studies demonstrated that butyrate is beneficial in modulating mucosal immunity, inflammatory response and intestinal barrier function (Tan et al., 2014; Yang et al., 2020). In particular, the supplementation of butyric acid induced a decrease of inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6) and increased anti-inflammatory

signalling molecules (IL-10 and TGF-β) (Byrne et al., 2015; Knudsen et al., 2018). Tributyrin and butyrate supplementations in calves, piglets and broilers showed to alleviate inflammation lowering the oxidative stress (Huang et al., 2015; Bortoluzzi et al., 2017; Liu et al., 2022).
Tributyrin compared to sodium butyrate presents some advantages due to its longer metabolic half-life, more efficient induction of host defence peptides, and absence of offensive odour which affects the feed intake (Miyoshi et al., 2011; Jiang et al., 2013; Hu et al., 2022). All these characteristics make tributyrin an interesting feed additive for animal nutrition.

1.5.4 Microbial derived-products and additives

Leonardite

Humic substances are part of the organic matter of the soil, water and sediment. They are derived from chemical reactions along the decay and transformation of plants and microbes. The process of humification transforms many compounds (carbohydrates, phenols, proteins) into large complex and polymeric structures. For these reasons, humic substances have a huge variability in their composition and structure. Humic substances can be classified into three main fractions based on their solubility: humic acids, fulvic acids and humin (de Melo et al., 2016). Recently, humic substances renewed a particular interest for their anti-inflammatory, immuno-modulatory, antioxidant and antimicrobial activities (Canellas and Olivares, 2014; Monda et al., 2017; Monda et al., 2018; Bernstein et al., 2019; Verrillo et al., 2022).
Among humic substances, leonardite is a microbially derived product mainly composed of humic substances, characterized by a high concentration of humic acids. Trckova et al. (2018) observed a significant reduction in diarrhoea severity and mortality, concomitantly with higher feed intake in weaned piglets after dietary supplementation of leonardite. The effectiveness of leonardite can considerably vary depending on the content of humic substances and their fractions. Even if its mechanism of action has not been fully understood, humic substances have an high affinity for biological membranes and they are involved in ion transport (Trckova et al., 2018). It was reported that after humic substances administration the improved animal performance were associate with an improved intestinal morphology, enhanced nutrient absorption, and modulation of gut microbiota (Islam et al., 2005; Wang et al., 2008; Chang et al., 2014). The antioxidant activity of leonardite is probably due to carboxylic and phenolic groups that constitutes humic acids, that may affect the oxidative status of the host (Khil'ko et al., 2011; Trckova et al., 2017).

Humic substances have capabilities to chelate minerals and increase their bioavailability. This characteristic have been explained with the presence of carboxyl and hydroxyl groups that contribute to the bioavailability of minerals in the body (Islam et al., 2005; Ipek et al., 2008). Leonardite supplementation increased serum levels of calcium, phosphorus and magnesium broilers (Samudovska and Demeterova, 2010). Other studies showed that leonardite supplementation may influence the lipid metabolism, but the exact mechanisms below this effect need to be further investigated (Wang et al., 2007; Macit et al., 2009; Trckova et al., 2018).
Leonardite is a natural source of humic substances that showed encouraging results regarding animal health and performance. However, more studies are needed to better clarify its mechanism of action and its optimal composition and concentration for feed formulations as source of bioactive compounds that can contribute to decrease antibiotics consumption.

Probiotics

The definition of probiotics was firstly formulated by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) in 2001 as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (Hotel and Cordoba, 2001). Most of probiotics belongs to *Lactobacillus* and *Bifidobacterium* genera as they are generally recognized as safe and commonly live in the GIT. Probiotics interact with both host and microbiota providing some beneficial effect due to their immunogenic potential, production of antimicrobial molecules, enhancement of mucosal integrity, competition for adhesion sites and nutrients with pathogens (Bermudez-Brito et al., 2012; Hill et al., 2014). The beneficial effect of probiotics is strictly related to the microbial strain, thus different multi-strain combinations have been developed to obtain a synergistic effect (Roselli et al., 2017). *Bacillus subutilis* showed to modulate inflammatory response and gut permeability. In addition, some strains of *Bacillus* demonstrated to improve animal performance and modulate gut microbiota (Rhayat et al., 2017; Wealleans et al., 2017).
Lactobacillus spp. is another genus commonly used as probiotic. Studies have shown that *Lactobacillus* strains have the ability to inhibit pathogens and decrease the inflammatory response (Bajagai et al., 2016; Buntyn et al., 2016; Mignacca et al., 2017). The enhancement of growth performance after probiotic supplementation has been confirmed through a higher feed digestibility, conversion rate, and production of volatile fatty acids (El-Tawab et al., 2016). Another important effect of probiotics is the antagonistic behaviour towards pathogens. Lactic acid bacteria, commonly used as probiotics, produce organic acids that decrease the pH in the GIT, lowering the

pathogenic strains survival and regulating the microbiota. *Enterococcus faecium* supplementation in piglets reduced the abundance of *Clostridium perfrigens* (Hanczakowska et al., 2016). Literature studies demonstrated that lactic acid bacteria supplementation reduced *Salmonella, Shigella* and *E. coli* presence in faeces and intestinal environment of goats and pigs (Apás et al., 2010; Chiang et al., 2015). Some probiotic strains are able to produce exopolysaccharides that inhibit biofilm formation, and they have been also employed as preservative for food products (Alayande et al., 2020). *Lactobacillus* spp. were shown to reduce the contamination of feed from mycotoxins detoxifying different forms of mycotoxin by cell wall adsorption and biotransformation (Wang et al., 2019b; Chlebicz and Śliżewska, 2020). Ran et al. (2019) evaluated the use of Lactobacillus-base products compared to in-feed antibiotics on performance, carcass characteristics and blood metabolites. Results shown that probiotic supplementation increased growth performance, feed efficiency and the treated group registered a lower number of antibiotic treatments thus confirming probiotics as potential alternatives to antibiotics. Probiotics supplementation in broilers shown to modulate lipid metabolism (cholesterol, triglycerides) and immunological parameters (Mujnisa et al., 2018; Vase-Khavari et al., 2019).

In weaned piglets, the supplementation of a probiotic mixture (*L. reuteri*, *L. salivarius* and *Streptococcus salivarius*) boosted growth performance, blood parameters and immunoglobulin G content (Aiyegoro et al., 2017). *L. salivarius* showed to inhibit the colonization of *E. coli* K88 and increase intestinal integrity *in vitro* (Yeo et al., 2016).

Considering the important concerns regarding AMR, probiotics have good chances as valid alternative to antibiotics in livestock due to their positive impact on animal health.

References

Adhikari, P., Yadav, S., Cosby, D.E., Cox, N.A., Jendza, J.A., Kim, W.K., 2020. Research Note: Effect of organic acid mixture on growth performance and Salmonella Typhimurium colonization in broiler chickens. Poultry science 99, 2645-2649.

Aiyegoro, O., Dlamini, Z., Okoh, A., Langa1, R., 2017. Effects of probiotics on growth performance, blood parameters, and antibody stimulation in piglets. South African Journal of Animal Science 47, 766-775.

Al-Alo, K., Brujeni, G.N., Lotfollahzadeh, S., Moosakhani, F., Gharabaghi, A., 2018. Correlation between neonatal calf diarrhea and the level of maternally derived antibodies. Iranian Journal of Veterinary Research 19, 3.

Aland, A., Madec, F., 2009. Sustainable animal production: the challenges and potential developments for professional farming. Wageningen Academic Publishers.

Alayande, K.A., Aiyegoro, O.A., Ateba, C.N., 2020. Probiotics in animal husbandry: Applicability and associated risk factors. Sustainability 12, 1087.

Allen, H.K., Levine, U.Y., Looft, T., Bandrick, M., Casey, T.A., 2013. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. Trends in microbiology 21, 114-119.

Alshaibani, D., Zhang, R., Wu, V.C., 2017. Antibacterial characteristics and activity of Vaccinium macrocarpon proanthocyanidins against diarrheagenic Escherichia coli. Journal of Functional Foods 39, 133-138.

AlSheikh, H.M.A., Sultan, I., Kumar, V., Rather, I.A., Al-Sheikh, H., Tasleem Jan, A., Haq, Q.M.R., 2020. Plant-based phytochemicals as possible alternative to antibiotics in combating bacterial drug resistance. Antibiotics 9, 480.

Amalaradjou, M.A.R., Narayanan, A., Venkitanarayanan, K., 2011. Trans-cinnamaldehyde decreases attachment and invasion of uropathogenic Escherichia coli in urinary tract epithelial cells by modulating virulence gene expression. The Journal of urology 185, 1526-1531.

Apak, R.a., Oʹzyuʹrek, M., Guʹcʸluʹ, K., Cʸapanogʹlu, E., 2016. Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. Journal of agricultural and food chemistry 64, 997-1027.

Apás, A.L., Dupraz, J., Ross, R., González, S.N., Arena, M.E., 2010. Probiotic administration effect on fecal mutagenicity and microflora in the goat's gut. Journal of bioscience and bioengineering 110, 537-540.

Avelar Rodriguez, D., Peña Vélez, R., Toro Monjaraz, E.M., Ramirez Mayans, J., Ryan, P.M., 2019. The gut microbiota: a clinically impactful factor in patient health and disease. SN Comprehensive Clinical Medicine 1, 188-199.

Bag, A., Bhattacharyya, S., Chattopadhyay, R., 2013. Isolation and identification of a gallotannin 1, 2, 6-tri-O-galloyl-β-d-glucopyranose from hydroalcoholic extract of T erminalia chebula fruits effective against multidrug-resistant uropathogens. Journal of Applied Microbiology 115, 390-397.

Baghban-Kanani, P., Hosseintabar-Ghasemabad, B., Azimi-Youvalari, S., Seidavi, A., Ragni, M., Laudadio, V., Tufarelli, V., 2019. Effects of using Artemisia annua leaves, probiotic blend, and organic acids on performance, egg quality, blood biochemistry, and antioxidant status of laying hens. The Journal of Poultry Science 56, 120-127.

Baird, L., Dinkova-Kostova, A.T., 2011. The cytoprotective role of the Keap1–Nrf2 pathway. Archives of toxicology 85, 241-272.

Bajagai, Y.S., Klieve, A.V., Dart, P.J., Bryden, W.L., 2016. Probiotics in animal nutrition: production, impact and regulation. FAO.

Bartoš, P., Dolan, A., Smutný, L., Šístková, M., Celjak, I., Šoch, M., Havelka, Z., 2016. Effects of phytogenic feed additives on growth performance and on ammonia and greenhouse gases emissions in growing-finishing pigs. Animal Feed Science and Technology 212, 143-148.

Belkaid, Y., Hand, T.W., 2014. Role of the microbiota in immunity and inflammation. Cell 157, 121-141.

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., Gil, A., 2012. Probiotic mechanisms of action. Annals of Nutrition and Metabolism 61, 160-174.

Bernstein, N., Gorelick, J., Zerahia, R., Koch, S., 2019. Impact of N, P, K, and humic acid supplementation on the chemical profile of medical cannabis (Cannabis sativa L). Frontiers in plant science 10, 736.

Binot, A., Duboz, R., Promburom, P., Phimpraphai, W., Cappelle, J., Lajaunie, C., Goutard, F.L., Pinyopummintr, T., Figuié, M., Roger, F.L., 2015. A framework to promote

collective action within the One Health community of practice: using participatory modelling to enable interdisciplinary, cross-sectoral and multi-level integration. One Health 1, 44-48.
Bogere, P., Choi, Y., Heo, J., 2019. Probiotics as alternatives to antibiotics in treating post-weaning diarrhoea in pigs. South African Journal of Animal Science 49, 403-416.
Borrelli, L., Varriale, L., Dipineto, L., Pace, A., Menna, L.F., Fioretti, A., 2021. Insect derived lauric acid as promising alternative strategy to antibiotics in the antimicrobial resistance scenario. Frontiers in Microbiology 12, 620798.
Bortoluzzi, C., Pedroso, A., Mallo, J., Puyalto, M., Kim, W., Applegate, T., 2017. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. Poultry science 96, 3981-3993.
Brewer, M., 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Comprehensive reviews in food science and food safety 10, 221-247.
Brunauer, M., Roch, F.-F., Conrady, B., 2021. Prevalence of worldwide neonatal calf diarrhoea caused by bovine rotavirus in combination with Bovine Coronavirus, Escherichia coli K99 and Cryptosporidium spp.: a meta-analysis. Animals 11, 1014.
Buntyn, J.O., Schmidt, T., Nisbet, D.J., Callaway, T.R., 2016. The role of direct-fed microbials in conventional livestock production.
Byrne, C., Chambers, E., Morrison, D., Frost, G., 2015. The role of short chain fatty acids in appetite regulation and energy homeostasis. International journal of obesity 39, 1331-1338.
Cakmak, K.C., Gülçin, İ., 2019. Anticholinergic and antioxidant activities of usnic acid-An activity-structure insight. Toxicology reports 6, 1273-1280.
Canani, R.B., Di Costanzo, M., Leone, L., 2012. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. Clinical epigenetics 4, 1-7.
Canellas, L.P., Olivares, F.L., 2014. Physiological responses to humic substances as plant growth promoter. Chemical and Biological Technologies in Agriculture 1, 1-11.
Caprarulo, V., Giromini, C., Rossi, L., 2020. Chestnut and quebracho tannins in pig nutrition: the effects on performance and intestinal health. Animal, 100064.
Chan, B.C., Ip, M., Lau, C.B., Lui, S., Jolivalt, C., Ganem-Elbaz, C., Litaudon, M., Reiner, N.E., Gong, H., See, R.H., 2011. Synergistic effects of baicalein with ciprofloxacin against NorA over-expressed methicillin-resistant Staphylococcus aureus (MRSA) and inhibition of MRSA pyruvate kinase. Journal of ethnopharmacology 137, 767-773.
Chan, C.-L., Gan, R.-y., Shah, N.P., Corke, H., 2018a. Enhancing antioxidant capacity of Lactobacillus acidophilus-fermented milk fortified with pomegranate peel extracts. Food bioscience 26, 185-192.
Chan, C.-L., Gan, R.-Y., Shah, N.P., Corke, H., 2018b. Polyphenols from selected dietary spices and medicinal herbs differentially affect common food-borne pathogenic bacteria and lactic acid bacteria. Food Control 92, 437-443.
Chan, C.L., Gan, R.Y., Corke, H., 2016. The phenolic composition and antioxidant capacity of soluble and bound extracts in selected dietary spices and medicinal herbs. International Journal of Food Science & Technology 51, 565-573.
Chang, Q., Lu, Z., He, M., Gao, R., Bai, H., Shi, B., Shan, A., 2014. Effects of dietary supplementation of fulvic acid on lipid metabolism of finishing pigs. Journal of animal science 92, 4921-4926.

Chiang, M.-L., Chen, H.-C., Chen, K.-N., Lin, Y.-C., Lin, Y.-T., Chen, M.-J., 2015. Optimizing production of two potential probiotic Lactobacilli strains isolated from piglet feces as feed additives for weaned piglets. Asian-Australasian journal of animal sciences 28, 1163.

Chlebicz, A., Śliżewska, K., 2020. In vitro detoxification of aflatoxin B1, deoxynivalenol, fumonisins, T-2 toxin and zearalenone by probiotic bacteria from genus Lactobacillus and Saccharomyces cerevisiae yeast. Probiotics and antimicrobial proteins 12, 289-301.

Cho, Y.-i., Yoon, K.-J., 2014. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. Journal of veterinary science 15, 1-17.

Choi, Y., Goel, A., Hosseindoust, A., Lee, S., Kim, K., Jeon, S., Noh, H., Kyong Kwon, I., Chae, B., 2016. Effects of dietary supplementation of Ecklonia cava with or without probiotics on the growth performance, nutrient digestibility, immunity and intestinal health in weanling pigs. Italian Journal of Animal Science 15, 62-68.

Christaki, E., Florou-Paneri, P., Bonos, E., 2011. Microalgae: a novel ingredient in nutrition. International journal of food sciences and nutrition 62, 794-799.

Cormican, M., Hopkins, S., Jarlier, V., Reilly, J., Simonsen, G., Strauss, R., Vandenberg, O., Zabicka, D., Zarb, P., Catchpole, M., Heuer, O., Iosifidis, E., Monnet, D., Plachouras, D., Weist, K., Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Escamez, P., Girones, R., Herman, L., Koutsoumanis, K., Lindqvist, R., Norrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlstrom, H., Tenhagen, B., Teale, C., Schuepbach, G., Beloeil, P., Liebana, E., Stella, P., Murphy, D., Hauser, B., Urbain, B., Kozhuharov, E., Bozic, F., Michaelidou-Patsia, A., Bures, J., Vestergaard, E., Baptiste, K., Tiirats, T., Nevalainen, M., Rouby, J., Hahn, G., Schlumbohm, W., Malemis, I., Kulcsar, G., Lenhardsson, J., Beechinor, J., Breathnach, R., Pasquali, P., Auce, Z., Maciulskis, P., Schmit, M., Spiteri, S., Schefferlie, G., Hekman, P., Bergendahl, H., Swiezcicka, A., Da Silva, J., Taban, L., Hederova, J., Straus, K., Madero, C., Persson, E., Jukes, H., Weeks, J., Kivilahti-Mantyla, K., Moulin, G., Wallmann, J., Grave, K., Greko, C., Munoz, C., Bouchard, D., Catry, B., Moreno, M., Pomba, C., Rantala, M., Ruzauskas, M., Sanders, P., Schwarz, C., van Duijkeren, E., Wester, A., Ignate, K., Kunsagi, Z., Torren-Edo, J., ECDC, BIOH, E.P.B.H., CVMP, E.C.M.P.V., 2017. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. Efsa Journal 15.

Correddu, F., Lunesu, M.F., Buffa, G., Atzori, A.S., Nudda, A., Battacone, G., Pulina, G., 2020. Can agro-industrial by-products rich in polyphenols be advantageously used in the feeding and nutrition of dairy small ruminants? Animals 10, 131.

Costa, L., Luciano, F., Miyada, V., Gois, F., 2013. Herbal extracts and organic acids as natural feed additives in pig diets. South African Journal of Animal Science 43, 181-193.

Dafale, N.A., Srivastava, S., Purohit, H.J., 2020. Zoonosis: an emerging link to antibiotic resistance under "one health approach". Indian journal of microbiology 60, 139-152.

de Melo, B.A.G., Motta, F.L., Santana, M.H.A., 2016. Humic acids: Structural properties and multiple functionalities for novel technological developments. Materials Science and Engineering: C 62, 967-974.

De Vries, H., Smidt, H., 2020. Microbiota development in piglets. The Suckling and Weaned Piglet; Farmer, C., Ed.; Wageningen Academic Publishers: Wageningen, The Netherlands, 179-205.

Dittoe, D.K., Ricke, S.C., Kiess, A.S., 2018. Organic acids and potential for modifying the avian gastrointestinal tract and reducing pathogens and disease. Frontiers in veterinary science 5, 216.

Domínguez Díaz, L., Fernández-Ruiz, V., Cámara, M., 2020. The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. Critical reviews in food science and nutrition 60, 1738-1746.

Dong, G., Liu, H., Yu, X., Zhang, X., Lu, H., Zhou, T., Cao, J., 2018. Antimicrobial and anti-biofilm activity of tannic acid against Staphylococcus aureus. Natural product research 32, 2225-2228.

Doyon, M., Labrecque, J., 2008. Functional foods: a conceptual definition. British Food Journal.

Duarte, M.E., Kim, S.W., 2022. Intestinal microbiota and its interaction to intestinal health in nursery pigs. Animal Nutrition 8, 169-184.

Dutot, M., Fagon, R., Hemon, M., Rat, P., 2012. Antioxidant, anti-inflammatory, and anti-senescence activities of a phlorotannin-rich natural extract from brown seaweed Ascophyllum nodosum. Applied biochemistry and biotechnology 167, 2234-2240.

Eberl, G., 2010. A new vision of immunity: homeostasis of the superorganism. Mucosal immunology 3, 450-460.

Ebrahimnejad, H., Burkholz, T., Jacob, C., 2014. Flavanols and proanthocyanidins, Recent advances in redox active plant and microbial products, Springer, pp. 211-232.

Eibl, C., Bexiga, R., Viora, L., Guyot, H., Félix, J., Wilms, J., Tichy, A., Hund, A., 2021. The Antibiotic Treatment of Calf Diarrhea in Four European Countries: A Survey. Antibiotics 10, 910.

Ekambaram, S.P., Perumal, S.S., Balakrishnan, A., 2016. Scope of hydrolysable tannins as possible antimicrobial agent. Phytotherapy Research 30, 1035-1045.

El Kolli, M., Laouer, H., El Kolli, H., Akkal, S., Sahli, F., 2016. Chemical analysis, antimicrobial and anti-oxidative properties of Daucus gracilis essential oil and its mechanism of action. Asian Pacific Journal of Tropical Biomedicine 6, 8-15.

El-Tawab, A., Youssef, I., Bakr, H., Fthenakis, G., Giadinis, N., 2016. Role of probiotics in nutrition and health of small ruminants. Polish Journal of Veterinary Sciences.

Elmastas, M., Celik, S.M., Genc, N., Aksit, H., Erenler, R., Gulcin, İ., 2018. Antioxidant activity of an Anatolian herbal tea—Origanum minutiflorum: isolation and characterization of its secondary metabolites. International journal of food properties 21, 374-384.

EMA, CVMP, CHMP, 2019. Categorisation of Antibiotics in the European Union. Answer to the Request from the European Commission for Updating the Scientific Advice on the Impact on Public Health and Animal Health of the Use of Antibiotics in Animals, European Medicines Agency Amsterdam, The Netherlands.

Emami, N.K., Daneshmand, A., Naeini, S.Z., Graystone, E., Broom, L., 2017. Effects of commercial organic acid blends on male broilers challenged with E. coli K88: Performance, microbiology, intestinal morphology, and immune response. Poultry science 96, 3254-3263.

Engels, C., Schieber, A., Gänzle, M.G., 2011. Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (Mangifera indica L.). Applied and Environmental Microbiology 77, 2215-2223.

Enzing, C., Ploeg, M., Barbosa, M., Sijtsma, L., 2014. Microalgae-based products for the food and feed sector: an outlook for Europe. JRC Scientific and policy reports, 19-37.

Erickson, P.S., Marston, S., Gemmel, M., Deming, J., Cabral, R., Murphy, M., Marden, J., 2012. Kelp taste preferences by dairy calves. Journal of dairy science 95, 856-858.

Etienne-Mesmin, L., Chassaing, B., Desvaux, M., De Paepe, K., Gresse, R., Sauvaitre, T., Forano, E., de Wiele, T.V., Schüller, S., Juge, N., 2019. Experimental models to study intestinal microbes–mucus interactions in health and disease. FEMS microbiology reviews 43, 457-489.

European Food Safety Authority (EFSA), 2015. EFSA Strategy 2020 Trusted science for safe food.

Evans, F., Critchley, A., 2014. Seaweeds for animal production use. Journal of applied phycology 26, 891-899.

Everaert, N., Van Cruchten, S., Weström, B., Bailey, M., Van Ginneken, C., Thymann, T., Pieper, R., 2017. A review on early gut maturation and colonization in pigs, including biological and dietary factors affecting gut homeostasis. Animal feed science and technology 233, 89-103.

FAO, 2018. World Livestock: Transforming the livestock sector through the Sustainable Development Goals. , Rome, p. 222 pp.

Farha, A.K., Yang, Q.-Q., Kim, G., Li, H.-B., Zhu, F., Liu, H.-Y., Gan, R.-Y., Corke, H., 2020. Tannins as an alternative to antibiotics. Food Bioscience 38, 100751.

FEFAC, 2020. The European Feed Manufacturers' Federation. Feed Sustainability Charter 2030.

Ferdous, U.T., Yusof, Z.N.B., 2021. Medicinal prospects of antioxidants from algal sources in cancer therapy. Frontiers in Pharmacology 12, 593116.

Food and Agricolture Organization (FAO), 2017. The future of food and agriculture-Trends and challenges. Rome, Italy.

Frazzini, S., Scaglia, E., Dell'Anno, M., Reggi, S., Panseri, S., Giromini, C., Lanzoni, D., Sgoifo Rossi, C.A., Rossi, L., 2022. Antioxidant and Antimicrobial Activity of Algal and Cyanobacterial Extracts: An In Vitro Study. Antioxidants 11, 992.

Gadde, U., Kim, W., Oh, S., Lillehoj, H.S., 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Animal health research reviews 18, 26-45.

Gahan, D., Lynch, M., Callan, J., O'sullivan, J., O'Doherty, J., 2009. Performance of weanling piglets offered low-, medium-or high-lactose diets supplemented with a seaweed extract from Laminaria spp. Animal 3, 24-31.

Galley, J.D., Yu, Z., Kumar, P., Dowd, S.E., Lyte, M., Bailey, M.T., 2014. The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor. Gut microbes 5, 748-760.

Geletu, U.S., Usmael, M.A., Bari, F.D., 2021. Rotavirus in calves and its zoonotic importance. Veterinary Medicine International 2021.

Gemeda, N., Tadele, A., Lemma, H., Girma, B., Addis, G., Tesfaye, B., Abebe, A., Gemechu, W., Yirsaw, K., Teka, F., 2018. Development, characterization, and evaluation of novel broad-spectrum antimicrobial topical formulations from Cymbopogon martini (Roxb.) W. Watson essential oil. Evidence-Based Complementary and Alternative Medicine 2018.

Gil-Gil, T., Ochoa-Sánchez, L.E., Baquero, F., Martínez, J.L., 2021. Antibiotic resistance: time of synthesis in a post-genomic age. Computational and Structural Biotechnology Journal 19, 3110-3124.

González-Ríos, H., Dávila-Ramírez, J., Peña-Ramos, E., Valenzuela-Melendres, M., Zamorano-García, L., Islava-Lagarda, T., Valenzuela-Grijalva, N., 2016. Dietary supplementation of ferulic acid to steers under commercial feedlot feeding conditions improves meat quality and shelf life. Animal Feed Science and Technology 222, 111-121.

Gouveia, L., Oliveira, A.C., 2009. Microalgae as a raw material for biofuels production. Journal of industrial microbiology and biotechnology 36, 269-274.

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L.D., Hidalgo, F.J., Miyashita, K., van Camp, J., Alasalvar, C., Ismail, A.B., 2018. Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? Food chemistry 264, 471-475.

Gulcin, İ., 2020. Antioxidants and antioxidant methods: An updated overview. Archives of toxicology 94, 651-715.

Gupta, A., Dwivedi, M., Mahdi, A.A., Nagana Gowda, G., Khetrapal, C.L., Bhandari, M., 2012. Inhibition of adherence of multi-drug resistant E. coli by proanthocyanidin. Urological research 40, 143-150.

Gutzeit, C., Magri, G., Cerutti, A., 2014. Intestinal IgA production and its role in host-microbe interaction. Immunological reviews 260, 76-85.

Gülcin, I., 2012. Antioxidant activity of food constituents: an overview. Archives of toxicology 86, 345-391.

Hanczakowska, E., Świątkiewicz, M., Natonek-Wiśniewska, M., Okoń, K., 2016. Medium chain fatty acids (MCFA) and/or probiotic Enterococcus faecium as a feed supplement for piglets. Livestock science 192, 1-7.

Heinritz, S.N., Mosenthin, R., Weiss, E., 2013. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. Nutrition research reviews 26, 191-209.

Heo, J.M., Opapeju, F., Pluske, J., Kim, J., Hampson, D., Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. Journal of animal physiology and animal nutrition 97, 207-237.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews Gastroenterology & hepatology.

Honda, K., Littman, D.R., 2016. The microbiota in adaptive immune homeostasis and disease. Nature 535, 75-84.

Hotel, A.C.P., Cordoba, A., 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Prevention 5, 1-10.

Hu, Q., Yin, F., Yang, L., Li, B., Lei, G., Wang, C., Yin, Y., Liu, D., 2022. Dietary tributyrin intervention improves the carcass traits, organ indices, and blood biomarker profiles in broilers under the isocaloric diets administration. Poultry Science, 102061.

Huang, C., Song, P., Fan, P., Hou, C., Thacker, P., Ma, X., 2015. Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. The Journal of nutrition 145, 2774-2780.

Humann, J., Lenz, L.L., 2009. Bacterial peptidoglycan-degrading enzymes and their impact on host muropeptide detection. Journal of innate immunity 1, 88-97.

Hyams, C., Camberlein, E., Cohen, J.M., Bax, K., Brown, J.S., 2010. The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infection and immunity 78, 704-715.

Ipek, H., Avci, M., Iriadam, M., Kaplan, O., Denek, N., 2008. Effects of humic acid on some hematological parameters, total antioxidant capacity and laying performance in Japanese quails. Archiv fur Geflugelkunde 72, 56-60.

Islam, K.M.S., Schuhmacher, A., Gropp, J.M., 2005. Humic Acid Substances in Animal Agriculture. Pakistan Journal of Nutrition 4, 126-134.

Jiang, W., Sunkara, L.T., Zeng, X., Deng, Z., Myers, S.M., Zhang, G., 2013. Differential regulation of human cathelicidin LL-37 by free fatty acids and their analogs. Peptides 50, 129-138.

Kalin, P., Gülçin, İ., Gören, A.C., 2015. Antioxidant activity and polyphenol content of cranberries (Vaccinium macrocarpon). Records of Natural Products 9, 496.

Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial cell factories 17, 1-21.

Khan, R.U., Chand, N., Akbar, A., 2016. Effect of organic acids on the performance of Japanese quails. Pakistan Journal of Zoology 48.

Khan, R.U., Naz, S., Raziq, F., Qudratullah, Q., Khan, N.A., Laudadio, V., Tufarelli, V., Ragni, M., 2022. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environmental Science and Pollution Research, 1-11.

Khanbabaee, K., Van Ree, T., 2001. Tannins: classification and definition. Natural product reports 18, 641-649.

Khawaskar, D.P., Sinha, D., Lalrinzuala, M.V., Athira, V., Kumar, M., Chhakchhuak, L., Mohanapriya, K., Sophia, I., Kumar, O., Chaudhuri, P., 2021. Pathotyping and antimicrobial susceptibility testing of Escherichia coli isolates from neonatal calves. Veterinary research communications, 1-10.

Khil'ko, S., Efimova, I., Smirnova, O., 2011. Antioxidant properties of humic acids from brown coal. Solid Fuel Chemistry 45, 367-371.

Kim, J.E., Lillehoj, H.S., Hong, Y.H., Kim, G.B., Lee, S.H., Lillehoj, E.P., Bravo, D.M., 2015. Dietary Capsicum and Curcuma longa oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. Research in veterinary science 102, 150-158.

Kim, S.-K., Himaya, S., 2011. Medicinal effects of phlorotannins from marine brown algae. Advances in food and nutrition research 64, 97-109.

Knudsen, K., Laerke, H., Hedemann, M., 2018. Impact of dietmodulated butyrate production on intestinal barrier function and inflammation., Nutrients, p. 1499.

Kolodziejczyk, A.A., Zheng, D., Elinav, E., 2019. Diet–microbiota interactions and personalized nutrition. Nature Reviews Microbiology 17, 742-753.

Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Bäckhed, H.K., Gonzalez, A., Werner, J.J., Angenent, L.T., Knight, R., 2012. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 150, 470-480.

Kumar, P., Chand, S., Maurya, P.K., 2016. Quercetin-modulated erythrocyte membrane sodium-hydrogen exchanger during human aging: correlation with ATPase's. Archives of physiology and biochemistry 122, 141-147.

Kyaw, B.M., Arora, S., Lim, C.S., 2011. Anti-Pseudomonal and anti-biofilm activity of tannic acid in combination with antibiotics. International Journal of Integrative Biology 11, 110.

Lallès, J.-P., Montoya, C.A., 2021. Dietary alternatives to in-feed antibiotics, gut barrier function and inflammation in piglets post-weaning: Where are we now? Animal Feed Science and Technology, 114836.

Lauridsen, C., Højberg, O., Kongsted, H., Canibe, N., 2017. A critical review on alternatives to antibiotics and pharmacological zinc for prevention of diarrhoea in pigs post-weaning. Rapport og vidensyntese, DCA 26.

Le, C.-F., Fang, C.-M., Sekaran, S.D., 2017. Intracellular targeting mechanisms by antimicrobial peptides. Antimicrobial agents and chemotherapy 61, e02340-02316.

Lee, M., Lin, W., Yu, B., Lee, T., 2017. Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals—A review. Asian-Australasian journal of animal sciences 30, 299.

Lewis, K., Ausubel, F.M., 2006. Prospects for plant-derived antibacterials. Nature biotechnology 24, 1504-1507.

Lillehoj, H., Liu, Y., Calsamiglia, S., Fernandez-Miyakawa, M., Chi, F., Cravens, R., Oh, S., Gay, C., 2018. Phytochemicals as antibiotic alternatives to promote growth and enhance host health. Veterinary Research 49.

Liu, J.-B., Ding, Y.-S., Zhang, Y., Chen, J.-B., Cui, B.-S., Bai, J.-Y., Lin, M.-B., Hou, Q., Zhang, P.-C., Li, S., 2015a. Anti-inflammatory hydrolyzable tannins from Myricaria bracteata. Journal of Natural Products 78, 1015-1025.

Liu, S., Chen, R., Hagedorn, C.H., 2015b. Tannic acid inhibits hepatitis C virus entry into Huh7. 5 cells. PLoS One 10, e0131358.

Liu, S., Wu, J., Wu, Z., Alugongo, G.M., Khan, M.Z., Li, J., Xiao, J., He, Z., Ma, Y., Li, S., 2022. Tributyrin administration improves intestinal development and health in pre-weaned dairy calves fed milk replacer. Animal Nutrition 10, 399-411.

Liu, Y., Espinosa, C.D., Abelilla, J.J., Casas, G.A., Lagos, L.V., Lee, S.A., Kwon, W.B., Mathai, J.K., Navarro, D.M., Jaworski, N.W., 2018. Non-antibiotic feed additives in diets for pigs: a review. Animal Nutrition 4, 113-125.

Lo Verso, L., Talbot, G., Morissette, B., Guay, F., Matte, J.J., Farmer, C., Gong, J., Wang, Q., Bissonnette, N., Beaulieu, C., 2020. The combination of nutraceuticals and functional feeds as additives modulates gut microbiota and blood markers associated with immune response and health in weanling piglets. Journal of animal science 98, skaa208.

Lopreiato, V., Mezzetti, M., Cattaneo, L., Ferronato, G., Minuti, A., Trevisi, E., 2020. Role of nutraceuticals during the transition period of dairy cows: A review. Journal of Animal Science and Biotechnology 11, 1-18.

Lorenzi, V., Muselli, A., Bernardini, A.F., Berti, L., Pagès, J.-M., Amaral, L., Bolla, J.-M., 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species. Antimicrobial agents and chemotherapy 53, 2209-2211.

Luise, D., Lauridsen, C., Bosi, P., Trevisi, P., 2019. Methodology and application of Escherichia coli F4 and F18 encoding infection models in post-weaning pigs. Journal of Animal Science and Biotechnology 10.

Lynegaard, J.C., Kjeldsen, N.J., Hansen, C.F., Williams, A.R., Nielsen, J.P., Amdi, C., 2022. Reduction in Diarrhoea and Modulation of Intestinal Gene Expression in Pigs Allocated a Low Protein Diet without Medicinal Zinc Oxide Post-Weaning. Animals 12, 989.

López-Andrés, P., Luciano, G., Vasta, V., Gibson, T.M., Biondi, L., Priolo, A., Mueller-Harvey, I., 2013. Dietary quebracho tannins are not absorbed, but increase the antioxidant capacity of liver and plasma in sheep. British Journal of Nutrition 110, 632-639.

Ma, N., Guo, P., Zhang, J., He, T., Kim, S.W., Zhang, G., Ma, X., 2018. Nutrients mediate intestinal bacteria–mucosal immune crosstalk. Frontiers in Immunology 9, 5.

Macit, M., Celebi, S., Esenbuga, N., Karaca, H., 2009. Effects of dietary humate supplementation on performance, egg quality and egg yolk fatty acid composition in layers. Journal of the Science of Food and Agriculture 89, 315-319.

Madeira, M.S., Cardoso, C., Lopes, P.A., Coelho, D., Afonso, C., Bandarra, N.M., Prates, J.A., 2017. Microalgae as feed ingredients for livestock production and meat quality: A review. Livestock Science 205, 111-121.

Maier, G.U., Breitenbuecher, J., Gomez, J.P., Samah, F., Fausak, E., Van Noord, M., 2022. Vaccination for the prevention of neonatal calf diarrhea in cow-calf operations: a scoping review. Veterinary and animal science, 100238.

Majumder, S., Sigamani, A., Rahman, T., Rahmatullah, M., 2020. Exploring phytochemicals as alternatives to antimicrobials-Prospects and potentials. World J. Pharm. Pharm. Sci 9, 1802-1813.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews 14, 217-232.

McCall, J., Hidalgo, G., Asadishad, B., Tufenkji, N., 2013. Cranberry impairs selected behaviors essential for virulence in Proteus mirabilis HI4320. Canadian journal of microbiology 59, 430-436.

McDonnell, P., Figat, S., O'doherty, J., 2010. The effect of dietary laminarin and fucoidan in the diet of the weanling piglet on performance, selected faecal microbial populations and volatile fatty acid concentrations. Animal 4, 579-585.

McEwen, S.A., Collignon, P.J., 2018. Antimicrobial resistance: a one health perspective. Microbiology spectrum 6, 6.2. 10.

Menichetti, B., Garcia-Guerra, A., Lakritz, J., Weiss, W., Velez, J., Bothe, H., Merchan, D., Schuenemann, G., 2021. Effect of timing of prepartum vaccination relative to pen change with an acidogenic diet on lying time and metabolic profile in Holstein dairy cows. Journal of Dairy Science 104, 11059-11071.

Mignacca, S.A., Dore, S., Spuria, L., Zanghì, P., Amato, B., Duprè, I., Armas, F., Biasibetti, E., Camperio, C., Lollai, S.A., 2017. Intramammary infusion of a live culture of Lactococcus lactis in ewes to treat staphylococcal mastitis. Journal of medical microbiology 66, 1798-1810.

Mishra, A., Sharma, A.K., Kumar, S., Saxena, A.K., Pandey, A.K., 2013. Bauhinia variegata leaf extracts exhibit considerable antibacterial, antioxidant, and anticancer activities. BioMed Research International 2013.

Miyoshi, M., Sakaki, H., Usami, M., Iizuka, N., Shuno, K., Aoyama, M., Usami, Y., 2011. Oral administration of tributyrin increases concentration of butyrate in the portal vein and prevents lipopolysaccharide-induced liver injury in rats. Clinical nutrition 30, 252-258.

Monda, H., Cozzolino, V., Vinci, G., Drosos, M., Savy, D., Piccolo, A., 2018. Molecular composition of the Humeome extracted from different green composts and their biostimulation on early growth of maize. Plant and Soil 429, 407-424.

Monda, H., Cozzolino, V., Vinci, G., Spaccini, R., Piccolo, A., 2017. Molecular characteristics of water-extractable organic matter from different composted biomasses and their effects on seed germination and early growth of maize. Science of the Total Environment 590, 40-49.

Morrow, A.L., Lagomarcino, A.J., Schibler, K.R., Taft, D.H., Yu, Z., Wang, B., Altaye, M., Wagner, M., Gevers, D., Ward, D.V., 2013. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. Microbiome 1, 1-16.

Mowat, A.M., Agace, W.W., 2014. Regional specialization within the intestinal immune system. Nature Reviews Immunology 14, 667-685.

Mujnisa, A., Gustina, L., Natsir, A., Hasan, S., 2018. Research Article Dosage Effects of Lactococcus lactis ssp. lactis 2 as a Probiotic on the Percentage of Carcass, Abdominal Fat Content and Cholesterol Level in Broilers.

Negi, P.S., 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International journal of food microbiology 156, 7-17.

Pancu, D.F., Scurtu, A., Macasoi, I.G., Marti, D., Mioc, M., Soica, C., Coricovac, D., Horhat, D., Poenaru, M., Dehelean, C., 2021. Antibiotics: Conventional Therapy and Natural Compounds with Antibacterial Activity—A Pharmaco-Toxicological Screening. Antibiotics 10, 401.

Papatsiros, V., Katsoulos, P.-D., Koutoulis, K., Karatzia, M., Dedousi, A., Christodoulopoulos, G., 2014. Alternatives to antibiotics for farm animals. CABI Reviews, 1-15.

Park, M., Cho, H., Jung, H., Lee, H., Hwang, K.T., 2014. Antioxidant and anti-inflammatory activities of tannin fraction of the extract from black raspberry seeds compared to grape seeds. Journal of food biochemistry 38, 259-270.

Parmar, A., Lakshminarayanan, R., Iyer, A., Mayandi, V., Leng Goh, E.T., Lloyd, D.G., Chalasani, M.L.S., Verma, N.K., Prior, S.H., Beuerman, R.W., 2018. Design and syntheses of highly potent teixobactin analogues against Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant enterococci (VRE) in vitro and in vivo. Journal of medicinal chemistry 61, 2009-2017.

Pieper, R., Tudela, C.V., Taciak, M., Bindelle, J., Pérez, J.F., Zentek, J., 2016. Health relevance of intestinal protein fermentation in young pigs. Animal health research reviews 17, 137-147.

Prieto, A., López-Novo, C., Díaz, P., Díaz-Cao, J.M., López-Lorenzo, G., Antón, C., Remesar, S., García-Dios, D., López, C., Panadero, R., 2022. Antimicrobial Susceptibility of Enterotoxigenic Escherichia coli from Diarrhoeic Neonatal Calves in Spain. Animals 12, 264.

Priyadarshani, I., Rath, B., 2012. Commercial and industrial applications of micro algae–A review. Journal of Algal Biomass Utilization 3, 89-100.

Purohit, H.J., 2018. Gut-bioreactor and human health in future. Indian journal of microbiology 58, 3-7.

Quosdorf, S., Schuetz, A., Kolodziej, H., 2017. Different inhibitory potencies of oseltamivir carboxylate, zanamivir, and several tannins on bacterial and viral neuraminidases as assessed in a cell-free fluorescence-based enzyme inhibition assay. Molecules 22, 1989.

Ran, T., Gomaa, W., Shen, Y., Saleem, A., Yang, W., McAllister, T., 2019. Use of naturally sourced feed additives (lactobacillus fermentation products and enzymes) in growing and finishing steers: Effects on performance, carcass characteristics and blood metabolites. Animal Feed Science and Technology 254, 114190.

Rasooly, R., Molnar, A., Choi, H.-Y., Do, P., Racicot, K., Apostolidis, E., 2019. In-vitro inhibition of staphylococcal pathogenesis by witch-hazel and green tea extracts. Antibiotics 8, 244.

Regulation EC 1831/2003, of the European Parliament and of the council, of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance).

Regulation EU 6/2019, Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC (Text with EEA relevance).

Rhayat, L., Jacquier, V., Brinch, K., Nielsen, P., Nelson, A., Geraert, P.-A., Devillard, E., 2017. Bacillus subtilis s train specificity affects performance improvement in broilers. Poultry Science 96, 2274-2280.

Rhouma, M., Fairbrother, J.M., Beaudry, F., Letellier, A., 2017. Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. Acta Veterinaria Scandinavica 59, 1-19.

Ricci, A., Olejar, K.J., Parpinello, G.P., Mattioli, A.U., Teslić, N., Kilmartin, P.A., Versari, A., 2016. Antioxidant activity of commercial food grade tannins exemplified in a wine model. Food Additives & Contaminants: Part A 33, 1761-1774.

Ricke, S., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry science 82, 632-639.

Rodrigues, F.F., Costa, J.G., Coutinho, H.D., 2009. Synergy effects of the antibiotics gentamicin and the essential oil of Croton zehntneri. Phytomedicine 16, 1052-1055.

Roselli, M., Pieper, R., Rogel-Gaillard, C., de Vries, H., Bailey, M., Smidt, H., Lauridsen, C., 2017. Immunomodulating effects of probiotics for microbiota modulation, gut health and disease resistance in pigs. Animal Feed Science and Technology 233, 104-119.

Rossi, L., Dell'Orto, V., Vagni, S., Sala, V., Reggi, S., Baldi, A., 2014. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic Escherichia coli strain in piglets. Veterinary Research Communications 38, 39-49.

Rossi, L., Di Giancamillo, A., Reggi, S., Domeneghini, C., Baldi, A., Sala, V., Dell'Orto, V., Coddens, A., Cox, E., Fogher, C., 2013. Expression of verocytotoxic Escherichia coli antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. Journal of Veterinary Science 14, 263-270.

Sahin, K., Orhan, C., Tuzcu, M., Ali, S., Sahin, N., Hayirli, A., 2010. Epigallocatechin-3-gallate prevents lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors in heat-stressed quails. Poultry Science 89, 2251-2258.

Salehi, B., Martorell, M., Arbiser, J.L., Sureda, A., Martins, N., Maurya, P.K., Sharifi-Rad, M., Kumar, P., Sharifi-Rad, J., 2018. Antioxidants: positive or negative actors? Biomolecules 8, 124.

Samudovska, A., Demeterova, M., 2010. Effect of diet supplemented with natural humic compounds and sodium humate on performance and selected metabolic variables in broiler chickens. Acta Veterinaria Brno 79, 385-393.

Saw, C.L.L., Yang, A.Y., Cheng, D.C., Boyanapalli, S.S.-S., Su, Z.-Y., Khor, T.O., Gao, S., Wang, J., Jiang, Z.-H., Kong, A.-N.T., 2012. Pharmacodynamics of ginsenosides: antioxidant activities, activation of Nrf2, and potential synergistic effects of combinations. Chemical Research in Toxicology 25, 1574-1580.

Schokker, D., Fledderus, J., Jansen, R., Vastenhouw, S.A., de Bree, F.M., Smits, M.A., Jansman, A.A., 2018. Supplementation of fructooligosaccharides to suckling piglets affects intestinal microbiota colonization and immune development. Journal of animal science 96, 2139-2153.

Scicutella, F., Mannelli, F., Daghio, M., Viti, C., Buccioni, A., 2021. Polyphenols and organic acids as alternatives to antimicrobials in poultry rearing: a review. Antibiotics 10, 1010.

Selim, S., Alfy, S., Al-Ruwaili, M., Abdo, A., Jaouni, S., 2012. Susceptibility of imipenem-resistant Pseudomonas aeruginosa to flavonoid glycosides of date palm (Phoenix dactylifera L.) tamar growing in Al Madinah, Saudi Arabia. African Journal of biotechnology 11, 416-422.

Serrano, J., Puupponen-Pimiä, R., Dauer, A., Aura, A.M., Saura-Calixto, F., 2009. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. Molecular nutrition & food research 53, S310-S329.

Settle, T., Leonard, S., Falkenstein, E., Fix, N., Van Dyke, K., Klandorf, H., 2014. Effects of a phytogenic feed additive versus an antibiotic feed additive on oxidative stress in broiler chicks and a possible mechanism determined by electron spin resonance. International journal of poultry science 13, 62.

Shah, J., 2003. The salicylic acid loop in plant defense. Current opinion in plant biology 6, 365-371.

Shahidi, F., Zhong, Y., 2015. Measurement of antioxidant activity. Journal of functional foods 18, 757-781.

Shanahan, F., van Sinderen, D., O'Toole, P.W., Stanton, C., 2017. Feeding the microbiota: transducer of nutrient signals for the host. Gut 66, 1709-1717.

Shin, N.-R., Whon, T.W., Bae, J.-W., 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology 33, 496-503.

Sindhi, V., Gupta, V., Sharma, K., Bhatnagar, S., Kumari, R., Dhaka, N., 2013. Potential applications of antioxidants–A review. Journal of pharmacy research 7, 828-835.

Smith, G., 2015. Antimicrobial decision making for enteric diseases of cattle. Veterinary Clinics: Food Animal Practice 31, 47-60.

Smith, G.W., Berchtold, J., 2014. Fluid therapy in calves. Veterinary Clinics: Food Animal Practice 30, 409-427.

Sukumar, M.R., König, B., 2018. Pomegranate extract specifically inhibits Clostridium difficile growth and toxin production without disturbing the beneficial bacteria in vitro. Infection and Drug Resistance 11, 2357.

Suresh, G., Das, R.K., Kaur Brar, S., Rouissi, T., Avalos Ramirez, A., Chorfi, Y., Godbout, S., 2018. Alternatives to antibiotics in poultry feed: molecular perspectives. Critical reviews in microbiology 44, 318-335.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L., 2014. The role of short-chain fatty acids in health and disease. Advances in immunology 121, 91-119.

Tang, Y., 2022. Nutraceuticals, p. 102265.

Tiedje, J.M., 2019. Environmental aspects of antibiotic resistance, Oxford University Press.

Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2017. Effects of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on the health, oxidative stress status and fatty acid profile in weaned piglets. Veterinarni Medicina 62, 16-28.

Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2018. The effect of leonardite and lignite on the health of weaned piglets. Research in Veterinary Science 119, 134-142.

Upadhyay, A., Johny, A.K., Amalaradjou, M.A.R., Baskaran, S.A., Kim, K.S., Venkitanarayanan, K., 2012. Plant-derived antimicrobials reduce Listeria monocytogenes virulence factors in vitro, and down-regulate expression of virulence genes. International Journal of Food Microbiology 157, 88-94.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K., 2014. Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. BioMed research international 2014.

Valenzuela-Grijalva, N.V., Pinelli-Saavedra, A., Muhlia-Almazan, A., Domínguez-Díaz, D., González-Ríos, H., 2017. Dietary inclusion effects of phytochemicals as growth promoters in animal production. Journal of animal science and technology 59, 1-17.

Vase-Khavari, K., Mortezavi, S.-H., Rasouli, B., Khusro, A., Salem, A.Z., Seidavi, A., 2019. The effect of three tropical medicinal plants and superzist probiotic on growth performance, carcass characteristics, blood constitutes, immune response, and gut microflora of broiler. Tropical animal health and production 51, 33-42.

Velasco, V., Williams, P., 2011. Improving meat quality through natural antioxidants. Chilean journal of agricultural research 71, 313.

Verrillo, M., Salzano, M., Savy, D., Di Meo, V., Valentini, M., Cozzolino, V., Piccolo, A., 2022. Antibacterial and antioxidant properties of humic substances from composted agricultural biomasses. Chemical and Biological Technologies in Agriculture 9, 1-15.

Walencka, E., Rozalska, S., Wysokinska, H., Rozalski, M., Kuzma, L., Rozalska, B., 2007. Salvipisone and aethiopinone from Salvia sclarea hairy roots modulate staphylococcal antibiotic resistance and express anti-biofilm activity. Planta medica 73, 545-551.

Wall, E.H., Doane, P.H., Donkin, S.S., Bravo, D., 2014. The effects of supplementation with a blend of cinnamaldehyde and eugenol on feed intake and milk production of dairy cows. Journal of Dairy science 97, 5709-5717.

Wang, N., Wu, W., Pan, J., Long, M., 2019. Detoxification strategies for zearalenone using microorganisms: A review. Microorganisms 7, 208.

Wang, Q., Chen, Y., Yoo, J., Kim, H., Cho, J., Kim, I., 2008. Effects of supplemental humic substances on growth performance, blood characteristics and meat quality in finishing pigs. Livestock Science 117, 270-274.

Wang, Q., Kim, H., Cho, J., Chen, Y., Yoo, J., Kim, I., 2007. Effects of supplemental humic substances on egg production and quality in laying hens, JOURNAL OF DAIRY SCIENCE, AMER DAIRY SCIENCE ASSOC 1111 N DUNLAP AVE, SAVOY, IL 61874 USA, pp. 519-519.

Wealleans, A., Sirukhi, M., Egorov, I., 2017. Performance, gut morphology and microbiology effects of a Bacillus probiotic, avilamycin and their combination in mixed grain broiler diets. British poultry science 58, 523-529.

WHO, 2019. World Health Organization. Critically important antimicrobials for human medicine.

WHO, 2022. World Health Organization. Antimicrobial resistance surveillance in Europe 2022–2020 data.

Whon, T.W., Kim, H.S., Shin, N.-R., Sung, H., Kim, M.-S., Kim, J.Y., Kang, W., Kim, P.S., Hyun, D.-W., Seong, H.J., 2021. Calf diarrhea caused by prolonged expansion of autochthonous gut Enterobacteriaceae and their lytic bacteriophages. Msystems 6, e00816-00820.

Windeyer, M., Leslie, K., Godden, S.M., Hodgins, D., Lissemore, K., LeBlanc, S., 2014. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Preventive veterinary medicine 113, 231-240.

Yadav, S., Jha, R., 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. Journal of animal science and biotechnology 10, 1-11.

Yang, I., Corwin, E.J., Brennan, P.A., Jordan, S., Murphy, J.R., Dunlop, A., 2016. The infant microbiome: implications for infant health and neurocognitive development. Nursing research 65, 76.

Yang, W., Yu, T., Huang, X., Bilotta, A.J., Xu, L., Lu, Y., Sun, J., Pan, F., Zhou, J., Zhang, W., 2020. Intestinal microbiota-derived short-chain fatty acids regulation of immune cell IL-22 production and gut immunity. Nature communications 11, 1-18.

Yeo, S., Lee, S., Park, H., Shin, H., Holzapfel, W., Huh, C.S., 2016. Development of putative probiotics as feed additives: validation in a porcine-specific gastrointestinal tract model. Applied microbiology and biotechnology 100, 10043-10054.

Yim, G., McClure, J., Surette, M.G., Davies, J.E., 2011. Modulation of Salmonella gene expression by subinhibitory concentrations of quinolones. The Journal of antibiotics 64, 73-78.

Yudiarti, T., Isroli, I., Yunianto, V., 2020. Nutritive and antioxidative properties of some selected agro-industrial by-products fermented with the fungus Chrysonillia crassa as alternative feedstuffs for poultry, Journal of Physics: Conference Series, IOP Publishing, p. 012145.

Zhang, L., Wu, W., Lee, Y.-K., Xie, J., Zhang, H., 2018. Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. Frontiers in microbiology 9, 48.

Zhang, R., Yang, S., An, Y., Wang, Y., Lei, Y., Song, L., 2022. Antibiotics and antibiotic resistance genes in landfills: A review. Science of The Total Environment 806, 150647.

Zhang, W.-X., Zhang, Y., Zhang, X.-W., Deng, Z.-X., Liu, J.-X., He, M.-L., Wang, H.-F., 2020. Effects of Dietary Supplementation with Combination of Tributyrin and Essential Oil on Gut Health and Microbiota of Weaned Piglets. Animals 10, 180.

2 Aim

The aim of the following project was to evaluate innovative functional feed additives for animal nutrition, in order to increase animal health, performance and reduce the use of antibiotics in farm animals. For reaching this goal the use of specific *in vitro, in vivo* and *ex vivo* approaches has been followed. In particular, the *in vitro* methodology was useful to (i characterize feed additives for their composition and functional properties, (ii screen different concentrations in order to optimize their inclusion, (iii obtain reliable data for decreasing the number of animals used for experimental purposes. *In vivo* trials were necessary to understand the effect of dietary inclusion of functional feed additives on (i zootechnical performance, ii) animal health and pathology occurrence, (iii gut microbiota modulation.

In addition, due to the constantly raising needing of science-based data and reducing or substituting animals for experimental purposes the research has been prompted to develop innovative models in line with 3R principles. For this reason, an *ex vivo* model was set up to study nutrient bioaccessibility and predict the nutritional value of ingredients and additives.

3 *In vitro* evaluation of innovative functional feed additives

Animal models have been used in research form the Greek Era. Documents from Alcmaeon of Croton have been found from in 6th century B.C documenting experiments with animals (Ericsson et al., 2013). Many models have been developed for different studies involving nematodes, flies, zebrafishes, frogs, mice, rats, rabbits, dogs and primates (Bédard et al., 2020).

The most used animal models are mice and rat. There are several reasons why animals are still used in research to study human diseases. Mice share 80% of genome with humans and they have a shorter life expectancy compared to humans, and scientist can study disease evolution a shorter lifetime than human being (Demetrius, 2006).

In 1959, Russel and Burch defined the 3R principles for animal use in research: replacement, reduction and refinement. The purpose of the principles was to offer better life quality to laboratory animals. The replacement is the primary aim. In several cases, animals models can be substituted by more accurate and innovative *in vitro* models (Bédard et al., 2020). In addition, the reproducibility in research studies that use animal models are considered lower. The replacement of animals can be pursued through different alternatives including *in vitro* protocols, tissue engineering and *ex vivo* tests. Cell culture is a promising approach complementary to animal experimentation or to completely substitute it. This model is an important tool for improving the knowledge on cell biology and different 2D and 3D methods have been developed (Meigs et al., 2018). Other alternatives involve enzymatic and microbiological systems, DNA and microfluidic chips (Arora et al., 2011).

Animal ethics is an important issue as human welfare. Even if it is not possible to completely substitute animal for experimental purposes, the use of *in vitro* studies has thus become valuable to acquire preliminary results that allow to reduce at the minimum the involvement of animals in research studies in line with the 3R principles. For this reason, the evaluation of functional characteristics of algae, tannins extract, and probiotic strains as innovative feed additives was firstly conducted *in vitro*.

References

Arora, T., Mehta, A., Joshi, V., Mehta, K., Rathor, N., Mediratta, P., Sharma, K., 2011. Substitute of animals in drug research: an approach towards fulfillment of 4R's. Indian journal of pharmaceutical sciences 73, 1.

Bédard, P., Gauvin, S., Ferland, K., Caneparo, C., Pellerin, È., Chabaud, S., Bolduc, S., 2020. Innovative human three-dimensional tissue-engineered models as an alternative to animal testing. Bioengineering 7, 115.

Demetrius, L., 2006. Aging in mouse and human systems: a comparative study. Annals of the New York Academy of Sciences 1067, 66-82.

Ericsson, A.C., Crim, M.J., Franklin, C.L., 2013. A brief history of animal modeling. Missouri medicine 110, 201.

Meigs, L., Smirnova, L., Rovida, C., Leist, M., Hartung, T., 2018. Animal testing and its alternatives–The most important omics is economics. ALTEX-Alternatives to animal experimentation 35, 275-305.

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In vitro evaluation of antimicrobial and antioxidant activities of algal extracts

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Brief Introduction to the Study:

The following study significantly contributed to my academic career. This scientific work laid the foundations for the development of the subsequent project funded by the University of Milan entitled: 'ASAP - Algae-based alternatives to in-feed antibiotics in pig production' (Seal of Excellence 2020) with Principal Investigator Prof. Luciana Rossi evaluating the effect of dietary supplementation of algae in post-weaning challenged piglets. The following research evaluated two important functional characteristics of *Ascophyllum nodosum* and *Schizochytrium* spp. as innovative feed ingredients. The algae tested showed dose-dependent antibacterial and antioxidant capacity depending on species and nutrient composition. The article is mainly based on the use of two *in vitro* techniques to evaluate the bioactivities of algae extracts, highlighting the potential for their use as sustainable functional ingredients for pig nutrition to reduce disease occurrence, improve animal health and reduce antibiotic use. Even if the *in vitro* results can not be directly translated to *in vivo*, the study demonstrated the potentiality of the investigated algal species and enriched the literature regarding innovative sustainable feed ingredients for pig nutrition. However, the importance of the paper relies on the fact that this manuscript was my first scientific publication, which allowed myself to gain valuable skills and experience in my journey as a young researcher going through the entire editorial and peer-review process.

3.1 *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts

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Abstract

The aim of this study was to evaluate the antioxidant capacity, the antimicrobial proprieties of algae *Ascophyllum nodosum* and *Schizochytrium* spp. against one of major swine enteric pathogen *Escherichia coli* O138 by broth macro-dilution method in Luria–Bertani (LB) medium. The antimicrobial effect of the algal extracts at supplementation of 0.12%, 0.06% and 0.03% (v/v) on *E. coli* O138, genetically characterised by PCR, was evaluated by following the bacterial growth. The antioxidant activity was determined by the ABTS Radical Cation Decolourisation Assay. In particular, the $\log_{10}$ *E. coli* used as control resulted significantly higher than 0.12% at 3 hours (8.82 ± 0.07 and 8.18 ± 0.07 $\log_{10}$ cells/mL, respectively; $p<.01$) suggesting an inhibitory activity related to the dose. No effect activity was observed with *Schizochytrium* spp. against *E. coli* growth. *A. nodosum* and *Schizochytrium* spp. exhibited antioxidant capacity ($p<.05$). The combination of them (1:1) exhibited antioxidant activity suggesting a synergistic effect ($p<.05$). The different proprieties of algal species that can modulate the O138 E. coli growth, one of the major pathogen of swine species, together with the antioxidant capacity, make them a promising functional feed additive to improve the gut health, therefore further studies are needed to confirm these activities *in vivo*.

Keywords: algae; antimicrobial; antioxidant; additives; functional feed.

Highlights

- The aim of the study was to evaluate the antimicrobial and antioxidant proprieties of two species of algae: *Ascophyllum nodosum* and *Schizochytrium* spp.
- *Ascophyllum nodosum* revealed antimicrobial effect against *Escherichia coli* O138. Both algae exhibited antioxidant capacity also with a synergistic effect.

Introduction

One of today's challenges, in line with the One-Health principles, is to reduce the use of drugs and antibiotics in humans and livestock because of the rise of antibiotic resistance (EFSA and ECDC 2013; Dhama et al. 2013). In this context, pig farming is one of the most profitable agricultural practices, however, antibiotics have often been used in order to deal with critical phases of a pig's life, such as weaning. Post Weaning Diarrhoea (PWD), a gastrointestinal disease mainly associated with certain *Escherichia coli* strains, represents the most common indication for the antimicrobial prescription (Amezcua et al. 2002). In particular, the pathogroup of porcine verocytotoxin-producing *Escherichia coli*, belonging to serogroups O138, O139, and O141, is characterized by a virulence profile responsible for acute and severe enterotoxaemia

and for important economic losses (Verdonck et al. 2002; Rossi et al. 2014). Many factors, infectious and non-infectious, are involved in the outbreak of the PWD that is considered a multifactorial disease where the nutrition plays a pivotal role (Rossi et al. 2013). The reduction of the use of antimicrobials in food-producing animals, replacing them where possible and re-thinking the livestock production system, is essential for the future of animal and public health (EFSA 2012; Murphy et al. 2017). For these reasons, several functional feed additives need to be evaluated in the weaning phase in order to increase the health status and reduce the need for antibiotics (Windisch et al. 2008). In this scenario algae, besides being a valid source of essential nutrients, can be a very interesting natural source of new compounds with biological activity that could be used as functional ingredients of pig rations (Makkar et al. 2016; Madeira et al. 2017).

Algae are a heterogeneous group of autotrophic, photosynthetic, aquatic organisms which ranges from single-celled microorganisms defined as microalgae, to huge complex seaweeds (Evans and Critchley 2014). *Schizochytrium* spp., a microalga from Thraustochytrids family, is one of the most commonly studied microalgae in animal nutrition because of its high content in n-3 and n-6 poly-unsaturated fatty acids with observed effects on animal's products quality and animal health status (Jiang et al. 2004; AbuGhazaleh et al. 2009; Madeira et al. 2017).

Macroalgae are macroscopic, multicellular plant-like organisms with a highly variable composition depending on species, habitat, temperature, light intensity, nutrients concentration of water (Makkar et al. 2016). Macroalgae can be divided into three groups: brown algae, red algae and green algae. Although brown algae are of lesser nutritional value than red and green algae, they contain important bioactive compounds. *Ascophyllum nodosum* is a large, common cold water brown alga and is one of the most used seaweeds in animal nutrition, it is rich in minerals, particularly potassium and iodine (Combet et al. 2014) and contains polyphenols and phlorotannins, enlisted as bioactive compounds (Munir et al. 2013; Makkar et al. 2016). Algal biomass can also have a positive impact on food security and the environmental impact: in fact, they are cheap to harvest and grow exclusively in water, preventing the use of arable land. They also release oxygen into the atmosphere and, if included in the diet of ruminants, are able to reduce methane emissions (Fievez et al. 2007). Algal culture technology is similar to terrestrial plants agriculture; however, algae possess higher productivity. Plants are in a different scale level with an evolved organization of tissues, but micro and macroalgae have some advantages compared to them: they are not cultivated in agricultural soils, they can grow in non-potable water, in coastal, arid and marginal areas unsuitable for agricultural purposes (Cardon et al. 2008; Gouveia et al. 2009; Bochenski et al. 2019). Moreover, considering the challenges

and the agrozootechnical scenario of the coming years, the enlargement of feed resource base through identification of novel feeds or development of new additives that enhance resources use efficiency would play an important role in sustainable development of the animal productions.

Even if the algae bioactivities have been recognized, considering the heterogeneity of the algae-based commercial products for feed and the intra- and interspecific differences among algae, it is necessary to evaluate their functional proprieties, define the suitable species and the possibility to combine them to enhance their effect. For these reasons, the aim of this study was to evaluate the *in vitro* antioxidant properties and the antibacterial effect against O138 *E. coli,* major pathogen in swine livestock, of *Ascophyllum nodosum* and *Schizochytrium* spp. in order to establish their possible further integration in weaned pigs' diets as functional feed additive and as possible alternative to antibiotic compounds.

Materials and methods

Evaluation of nutritional value

Algae dried meal samples were obtained from Italfeed S.r.l. (Milan, Italy). The samples were analysed for the main nutrient components (ether extract, crude proteins, fats, crude fiber, ash) according to AOAC "Official methods of analysis" in double.

Dry matter (DM) was obtained by inserting samples in preweigthed aluminium bags and dried in a forced-air oven at 105°C for 24 h (AOAC method 930.15).

Ashes (Ash) were obtained using a muffle furnace at 550°C (AOAC method 942.05). Crude protein (CP) was determined by a Kjeldahl method (AOAC method 2001.11). Ether extract (EE) was determined using ether extraction in the Soxtec system (DM 21/12/1998). Crude fiber (CF) was determined by filtering bag technique (AOCS method Ba 6a-05). Mineral content was determined after sample mineralization. In particular, pulverized samples (0.3 g) were digested by a microwave digestor system (Anton Paar MULTIWAVE-ECO) in Teflon tubes filled with 10 ml of 65% HNO_3 by applying a one-step temperature ramp (at 120°C in 10 min and maintained for 10 min). The mineralized samples were cooled for 20 minutes, and they were transferred into the polypropylene test tubes. Mineralized samples were diluted 1:100 with 0.3 M HNO_3 in MilliQ Water and the concentration of elements was measured by inductively coupled plasma atomic emission spectrophotometer (ICP; model: Optima 3300 XL, PerkinElmer Inc., Massachusetts, USA).

Table 1. Primers used for polymerase chain reaction (PCR). FedF gene is an essential adhesion protein of F18 fimbriae, VT2eB is the gene codifying verocytotoxin type 2 variant B-subunit.

Primers	Nucleotide sequences
FedF-5′	CCATGGCTACTCTACAAGTAGACAAGTCTGTTTC
FedF-3′	GAGCTCTTACTGTATCTCGAAAACAATGGGCACCG
VT2e-B subunit-5′	GGATCCATGAAGAAGATGTTTATAGCGG
VT2e-B subunit-3′	AACGGGTCCACTTCAAATGATTCTCGAG

Escherichia coli characterization

The O138 *E. coli* strain was obtained from the Lombardy and Emilia Romagna Experimental Zootechnic Institute (IZSLER, Italy).

The strain was genetically characterized for the presence of genes codifying for two virulence factors represented by the F18 adhesive fimbriae and the verocytotoxin (VT2e). In particular, specific oligonucleotides were designed for the detection of FedF gene essential for F18 adhesion fimbriae and the B subunit of VT2e, responsible for binding the toxin to the intestinal cell surface before the absorption (Table 1).

Genomic DNA was extracted using phenol/chloroform (1:1) from an overnight culture of *E. coli* strain and the quality of DNA was evaluated spectrophotometrically (260/280 ratio) and by agarose gel electrophoresis (0.8%, 10 V/cm 2 h) for quantification and to test for the presence of RNA or degraded DNA. The presence of FedF and VT2eB genes was evaluated by polymerase chain reaction (PCR) using specific primer-pairs. PCR were performed using the following experimental conditions: first denaturation 94°C for 2 minutes, denaturation phase 94°C for 1 minute, followed by annealing phase 55°C for 2 minutes and elongation phase 72°C for 2 minutes (the cycle was repeated 34 times). The volume of reaction mixture was 50µl, with 5µl of template (bacterial DNA) added to PCR mixture.

Antimicrobial assay

Antimicrobial extracts from algae *Ascophyllum nodosum* and *Schizochytrium* spp. were obtained according to Jiménez et al. (2009) method. Five grams of dried algal meal sample were dissolved in 150 mL of acetone and extracted using a Soxhlet apparatus for 6 hours. After the evaporation of the solvent under vacuum at 50°C, the residue (120 mg) was resuspended in 20 mL of MilliQ water, filtered with 0.22 µm syringe filter and stored at -20°C until the analysis.

A liquid culture-based growth inhibition assay with *E. coli* O138 was performed to evaluate the ability of algae Soxhlet extracts to inhibit bacterial growth. An overnight culture of *E. coli* O138 in Luria-Bertani (LB) liquid medium was used as the inoculum for the experiments.

The experiment was set up as follows: 10mL of LB with 120μL of *E. coli* culture in liquid LB medium without algal extract were used as a positive control in order to evaluate the bacterial growth without any external influence. Three concentrations of the algal extract were added to a 50 mL tube with 10 mL of LB, to obtain a final concentration of treatment of 0.12%, 0.06% and 0.03% respectively. These concentrations were tested to evaluate if lower concentrations, compared with previous literature (Jimenez et al, 2009), were able to exert antimicrobial activity also in order to optimize the possible future inclusion of algae in feed and considering their cost-effective.

120 μL of overnight LB culture of *E. coli* were then added to each 50 mL tube. The same algal extract concentrations were added to 10 mL of LB without adding 120 μL of *E. coli* culture for the negative controls. All the samples were then maintained at 37°C in a shaking incubator for six hours. The growth rate of *E. coli* was estimated, every hour for six hours, measuring the absorbance with a spectrophotometer (V-630 UV-VIS Spectrophotometer, JASCO, Germany) at an optical density (OD) of 600 nm.

The measured OD were converted in $\log_{10}$ of the number of cells/mL considering 1 OD = 1×10^9 cells/mL (Myers et al. 2013).

All assays were performed in technical duplicate and three biological replicates that are meant to verify the replicability of the experiment, using the same procedures repeating the experiment starting from the sample also repeating the test in different days.

Evaluation of antioxidant properties (ABTS assay)

To perform the antioxidant assay, dried meal of *Schizochytrium* spp. and *A. nodosum* were extracted using ethanol and water according to the method proposed by Machu et al. (2015) with some adaptations. One gram of each alga was dissolved in 10 mL of solvent (pure ethanol or water) and stirred for 24 hours at 23°C. A mixture of both algae was prepared using 1:1 (w/w) of *A. nodosum* and *Schizochytrium* spp. powder extracted with 10 mL of water or ethanol as solvents. The mixture was stirred for 24 hours at 23°C. Extracts were centrifugated for 10 min at 3000 rpm, the supernatants were collected and filtered with 0.45 μm syringe filter and stored at -20°C until the analysis. The antioxidant activity was tested by adopting an ABTS assay, according to Re et al. (1999). The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation was generated by the reaction of 7 mM ABTS with 2.45 mM of K-persulfate. The reaction mixture was left to stand in the dark for 16 hours at room temperature and used within two days. Working solutions of $ABTS^{\bullet+}$ were obtained by diluting $ABTS^{\bullet+}$ in ethanol in order to obtain an absorbance of 0.700 ± 0.02 OD at 734 nm at room temperature. First, a calibration curve was obtained using different

concentrations (2000 μM, 1500 μM, 1000 μM, 500 μM, 100 μM, 0 μM) of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as standard. The assay was performed using 10 μL of diluted sample added to 1 mL of working solution ($ABTS^{\bullet+}$). The absorbance was recorded from 1 to 6 minutes and all determinations were performed in triplicate. The *A. nodosum* and *Schizochytrium* spp. extracts were diluted in their solvents (water and ethanol) and tested in the following concentrations: 10%, 5%, 2%. Secondary, in order to test the synergistic effect, the mixture with an equal concentration of both algae (1:1 w/w) was then tested with the same concentrations: 10%, 5%, 2%. The concentrations tested were prepared diluting the original extract 1:5 (v/v), 1:10 (v/v) and 1:20 (v/v) in line with the range tested by Jimenez et al. (2009) for DPPH assay. The dilutions were also necessary to obtain absorbances comparable with the calibration curve in order to quantify the final results that were expressed as equivalent concentration of Trolox/g after six minutes (TroloxEq/g).

The total antioxidant capacity was expressed as the percentage inhibition (PI), according to the equation: $PI = [(AbsABTS^{\bullet+} - Abs\ sample) / AbsABTS^{\bullet+}] \times 100$; here $AbsABTS^{\bullet+}$ denotes the initial absorbance of diluted $ABTS^{\bullet+}$, and Abs sample denotes the absorbance of the sample in every 6 min of reaction.

Statistical Analysis

Data of antimicrobial and antioxidant assays were analysed using Proc GLIMMIX of SAS 9.4 (SAS Inst. Inc., Cary, NC). The model included the fixed effect of treatments, time, the interaction between treatment and time. Within significant two-way interactions, slice option was used to separate means within a specific treatments and time and the results are reported as least squares means (LSMEANS) and standard error. Data of μmol TroloxEq/g were compared using Proc GLM. The model included the effect of algae and extraction methods (water and ethanol) and the interaction between algae and extraction methods. Means were considered different when $p < .05$ and tended to different if $0.05 < p \leq 0.10$. Tukey-Kramer studentized adjustments were used to separate the means of extraction methods within the two-way interactions. Results are reported as means and standard deviations.

Results and Discussion

In this study, the attention was focalized on the *in vitro* evaluation of nutraceutical properties of *Schizochytrium* spp. and *Ascophyllum nodosum* in order to establish their further use as functional additives.

The obtained results of chemical analysis of algae are in line with literature (Makkar et al. 2016) and the commercial feed label (Table 2). Both algae were freeze-dried meal with moisture content less than 8% in order to guarantee adequate storage conditions.

In general, *A. nodosum* is characterized by a high content of minerals (more than 20% DM), in particular it is characterized by a high content of calcium and a low content of phosphorous. This aspect should be considered in the diet's formulation in order to maintain a correct balance of these two elements. However, if algae are used as feed additives, they will be included less than 5% in the diet and this percentage should not constitute an important change in the mineral balance. In general, mineral premix additive is always included during the ration formulations, the amount of minerals contained in algae must be considered for their enclosure in the feed in order to respect the admitted levels of European Union regulation (Reg 1081/2003/EC). In particular, some minerals are required as nutrients for the piglets, but they are frequently integrated in excess also to increase animal performances and this aspect could represent a risk for the environment (Hejna et al. 2018). Minerals such as Cadmium (Cd) are undesirable compounds and Cd could become toxic in higher concentrations. Our results revealed an amount of Cd in line with permitted level of European regulation about feed additives (Dir 2002/32/EC). Other minerals like zinc (Zn), copper (Cu) and selenium (Se) are useful to satisfy the requirements of animals, their concentration should be balanced with the mineral content of the ingredients utilized in animal feed, also respecting the safety of the environment (Table 3). On the contrary, *Schizochytrium* spp. contains a lower amount of minerals (5.25% DM), but it represents an important source of lipids. *A. nodosum* is characterized also by 8.25% (DM) of crude protein content with high biological value (Becker, 2004). Even if the amount of protein is comparable to the corn meal, it should be considered that the presence of non-protein nitrogen in different algal species can affect the results slightly. In particular, the quantification of the crude protein using the standard conversion value for nitrogen (6.25) could lead to an overestimation of the protein content if non-protein nitrogen is present (Lourenço et al. 1998; Lourenço et al. 2002).

Table 2. Chemical composition of dried algae samples of *A. nodosum* and *Schizochytrium* spp.

	Ascophyllum nodosum	*Schizochytrium* spp.
DM (%)	92.12	99.39
Ash (%)	21.41	5.26
CP (%)	8.25	0.00
EE (%)	3.31	25.33
CF (%)	3.57	0.00

All values are expressed as percentage of dry matter (% DM).
Dry matter (DM), Ashes (Ash), Crude Protein (CP), Ether Extract (EE), Crude Fiber (CF).

Table 3. Mineral composition of dried algae samples *A. nodosum* and *Schizochytrium* spp.

	A. nodosum	*Schizochytrium spp.*
Ca	10466.00	n.d.
Cd	0.49	0.36
Cu	4.19	n.d.
Fe	433.00	11.10
Mg	10546.20	15.40
Mn	132.00	10.10
P	51.90	48.50
Se	2.30	2.00
Zn	32.60	6.10

All values are expressed as mg/kg of fresh weight.
n.d. = not detectable

The antimicrobial properties were evaluated against O138 *E. coli,* one of the major enteric pathogens of weaned piglets responsible for postweaning enteritis and enterotoxaemia, that causes significant morbidity and mortality in pigs worldwide. PWD represents an important issue in swine farming that causes important economic losses, at the same time affecting the health of animals and leading to the consumption of antibiotic drugs (Rossi et al. 2013). The pathogenicity is usually influenced by the presence of virulence factors, such as VT2e toxin and the F18 adhesive fimbriae (Verdonck et al. 2002). The detection of FedF adhesion factor of F18 and of VTe2B gene confirmed the virulence profile of the O138 *E. coli* strain (Figure 1).

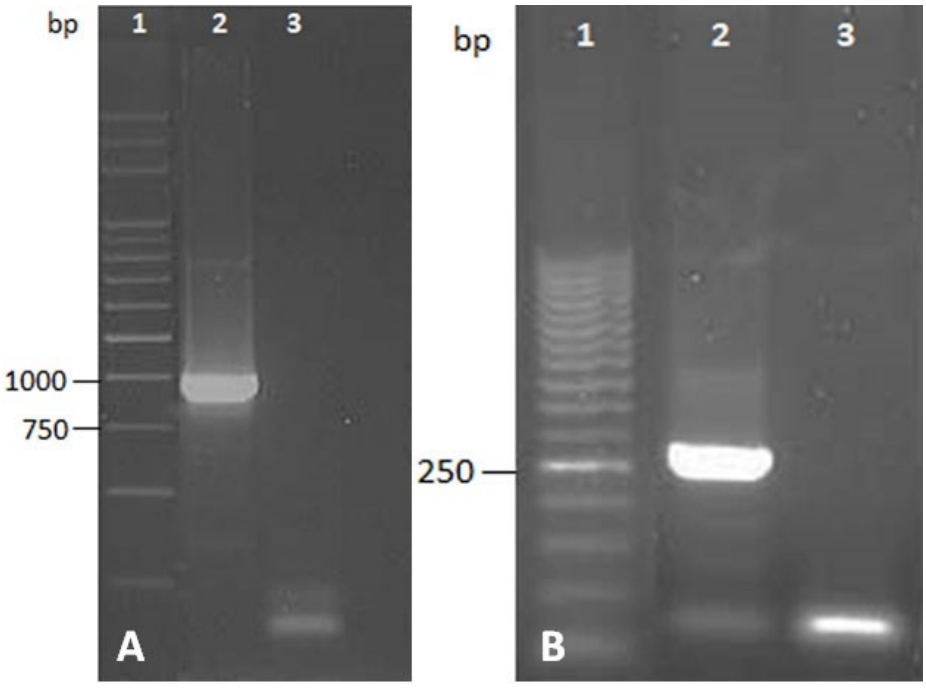

Fig. 1: (A) Agarose gel (1%) one-dimensional electrophoresis of PCR products for the detection of FedF gene from genomic DNA of O138 *Escherichia coli* strain. Lane 1: marker 1 kb; lane 2: positive sample;

lane 3: negative control sample. (B**)** Agarose gel (1.5%) one-dimensional electrophoresis of PCR products for the detection of VT2eB gene from genomic DNA of O138 *Escherichia coli* strain. Lane 1: marker 50 pb; lane 2: positive sample; lane 3: negative control sample.

Significant differences in the bacterial growth were observed among the *A. nodosum* concentrations, respectively: 0.12%, 0.06%, 0.03% and 0% (*E. coli* without treatment) ($p < .01$) (Figure 2). In particular, *A. nodosum* disclosed that the most concentrated treatment (0.12%) exhibited the highest inhibition activity on O138 *E. coli* growth; on the contrary, the lowest concentrations (0.06% and 0.03%) revealed a bacterial growth comparable to the positive control, indicating that these concentrations did not influence *E. coli* growth.

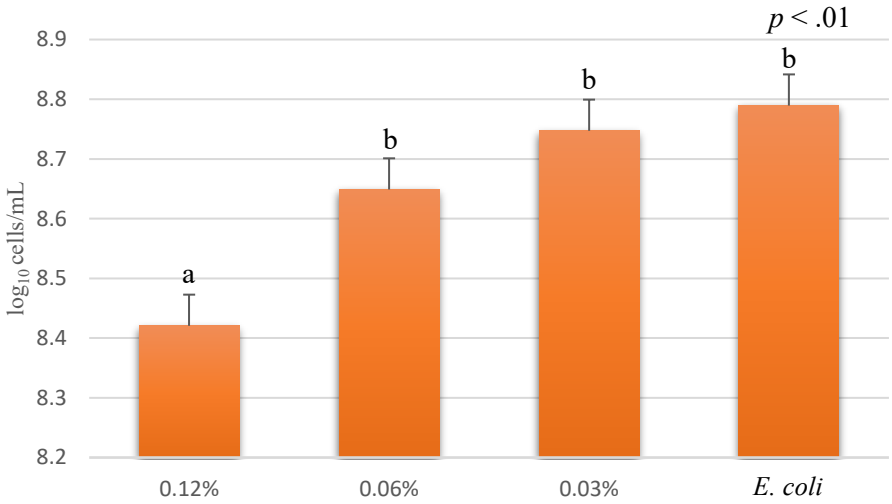

Figure 2: Average of *E. coli* growth (log_{10} cells/mL) of different concentrations of *A. nodosum* Soxhlet extract from time 1 to 6 hour. *A. nodosum* Soxhlet extract concentrations tested were 0.12%, 0.06%, 0.03% and positive control (*E. coli*).
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different (treatment $p < .01$).

Our results are in line with other findings, demonstrating a significant decrease in bacterial growth *in vitro* (Dierick et al. 2009). Nevertheless, obtained results confirmed the antimicrobial activity also against a wild type strain characterized by a relevant virulence profile.

The inhibitory effect is probably attributed to phlorotannins, which are known to be powerful bacteriostatic and also a bactericidal component of brown algae (Wang et al. 2009). Other components of brown seaweed have demonstrated to possess an antibacterial activity, in particular polyphenols, which are a class of secondary metabolites also known for their antimicrobial activity (Daglia, 2012). It has been demonstrated that polyphenols form different sources are able to produce hydrogen peroxide in aerobic conditions, its production is also directly related to the content of

the hydroxyl groups. Tannins possess affinity for bounding proteins and this capacity increases with the number of hydroxyl groups this could explain that phlorotannins exert higher antimicrobial activity compared with terrestrial tannins because they are readily oxidized upon exposure to air and contain a higher number of hydroxyl groups (Wang et al. 2009).

Our findings highlight the need to use the highest concentration in order to guarantee the antimicrobial effect. In particular, considering that the extract used in our study was obtained using 5 g of alga in 150 mL of acetone, obtaining 120 mg of solid extract that was then dissolved in 20 mL of water, the final concentration of algal extract resuspended in water was 0.6%. Our study is in line with Gardiner et al. (2007), which used an inclusion percentage of *A. nodosum* extract in piglet's diet ranging from 0.3% to 0.9% of dry matter. The effects of the different concentrations of algae tested to evaluate their inhibition capacity over time (Figure 3) revealed that after the first hour, the inhibition capacities of the tested concentrations were comparable to the positive control (*E. coli*). After 2, 3 and 4 hours, there was a significant difference among the most concentrated treatment (0.12%) and the other samples, which were comparable to the positive control (*E. coli*) ($p < .05$). The maximum inhibitory effect of algal extract was observed after 3 hours, were the $\log_{10}$ cells/mL of control (*E. coli*) and 0.12% concentration were significantly different, respectively 8.82±0.07 and 8.18±0.07 ($p < .01$). No differences were observed at 5 and 6 hours, suggesting an exhaustion of the bioactivity due to algal degradation or to the development of bacterial resistance. According to Zoetendal et al. (2008) the bacterial resistance of *E. coli* against condensed tannins seems to be related to the activation the BaeSR two-component regulatory system. Phlorotannins contained in *A. nodosum* possesses similar property to polyphenols, main constituent of tannins, characterised by antimicrobial activity.

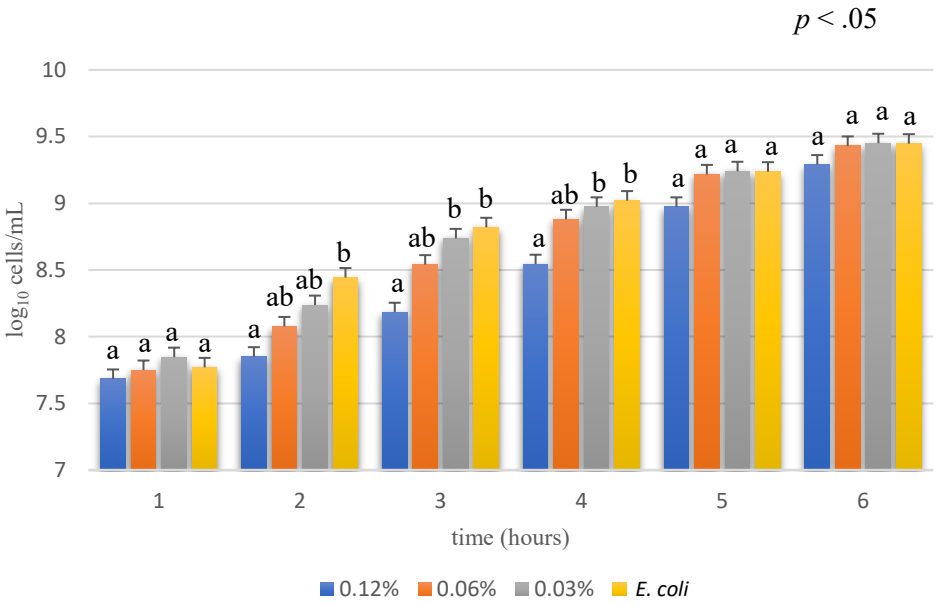

Figure 3: Average of *E. coli* growth (log_{10} cells/mL) of different concentration of *A. nodosum* Soxhlet extract through experimental time (from 1 to 6 hour). *A. nodosum* Soxhlet extract concentrations tested were 0.12%, 0.06%, 0.03% and positive control (*E. coli*).
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different, means are separated within treatment groups though the experimental time with Tukey (interaction treatment by time $p < .05$).

Even if the effect was observed only for a short period, it is important to consider the transit time of the feed in a pig's gastrointestinal tract, which lasts 4 hours, this guaranties the effect throughout the digestion tract.
Results of ABTS assay revealed that *A. nodosum* ethanol extract antioxidant activity (0.75±0.31 μmol TroloxEq/g) is lower compared to the water extract ($p < .05$) (Table 4) suggesting that this algal species contains a good amount of hydro-soluble antioxidant substances which are easily released in water (Figure 4). In line with our results, the study conducted by Machu et al. (2015) disclosed the highest antioxidant capacity adopting the water extraction for brown algae. Machu et al. (2015) tested *Eisenia bicyclis* that is a brown alga rich in phlorotannins that revealed also antimicrobial capacities against streptomycin resistant *Listeria monocitogenes* (Kim et al. 2017).

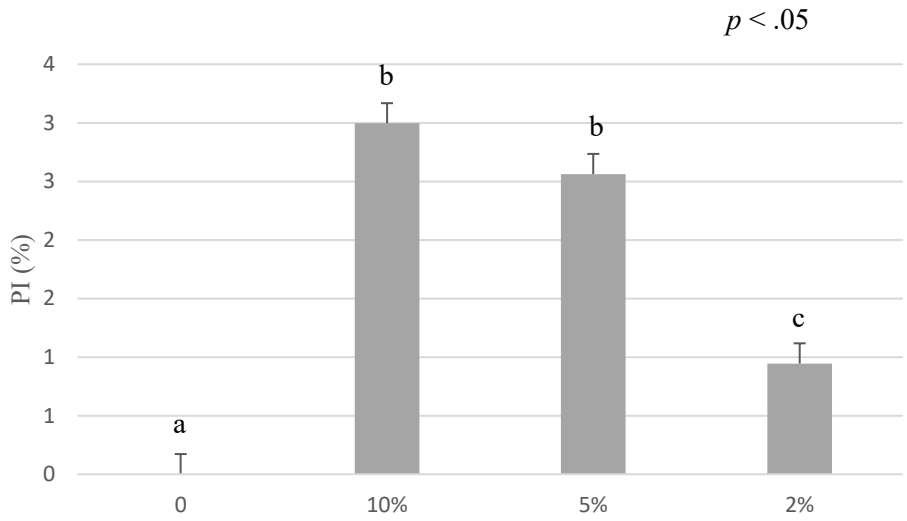

Figure 4: Average of percentage of inhibition (PI%) of *A. nodosum* ethanol extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* ethanol extract 10%, 5%, 2% and blank.
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different (treatment $p < .05$).

Table 4. Results of ABTS assay of *A. nodosum*, *Schizochytrium* spp. and the algal mixture (1:1 w/w) in response to the different extraction methods (water and ethanol). Results are expressed as µmol TroloxEq/g.

	Extraction methods	
	Water	Ethanol
A. nodosum	55.54±16.05[a]	0.75±0.31[b]
Schizochytrium spp.	n.d.	2.56±0.53
Mixture (1:1 w/w)	10.06±2.73[a]	3.18±0.57[b]

Data are shown as means and standard deviations.
a-b Means (n=3) with different superscripts are significantly different (Treatment $p < .05$).
n.d. = not detectable

The results have also displayed that the antioxidant activity of *A. nodosum* water extract exhibited an antioxidant activity of 55.54±16.05 µmol TroloxEq/g after 6 minutes of reaction (Table 4) (Figure 5). In general, free antioxidant substances react immediately when the sample is added to an $ABTS^{\bullet+}$ reaction mixture in a similar way to the effect of standard Trolox. Other antioxidants that are not immediately available may require time to be released in order to exert their effect against radicals, for this reason the assay was conducted in six minutes.

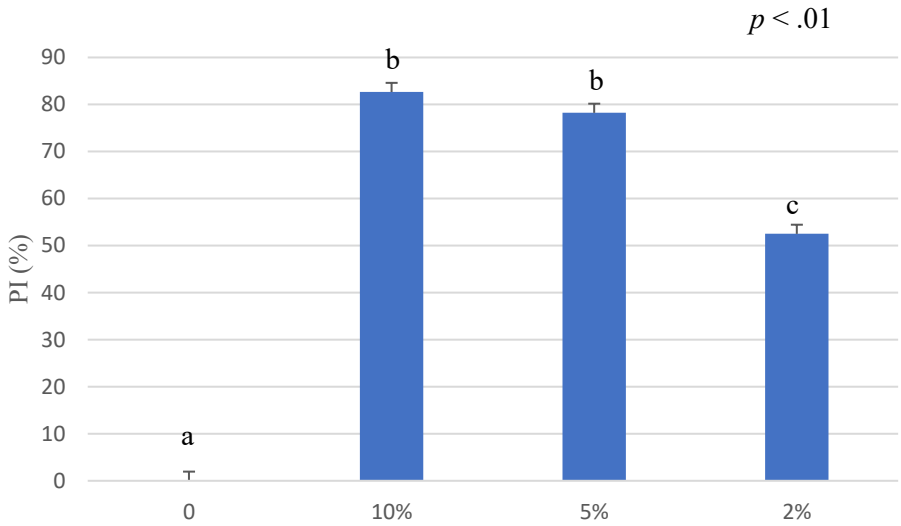

Figure 5: Average of percentage of inhibition (PI%) of *A. nodosum* water extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* water extract 10%, 5%, 2% and blank.
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different (treatment $p < .01$).

The *Schizochytrium* spp. ethanol extract exhibited an antioxidant capacity of 2.56±0.53 µmol TroloxEq/g calculated after six minutes of reaction (Table 4) (Figure 6). The *Schizochytrium* spp. water extract did not display any activity, in fact we obtained an opaque extract which was not able to inhibit the $ABTS^{\bullet+}$ radical. Furthermore, this extract when added to the working solution increased the turbidity of the mixture making the spectrophotometer reading inaccurate. These findings could be due to the

high content of lipids of *Schizochytrium* spp. (Table 2) that are known to be not hydro-soluble and water was not able to extract non-polar substances.

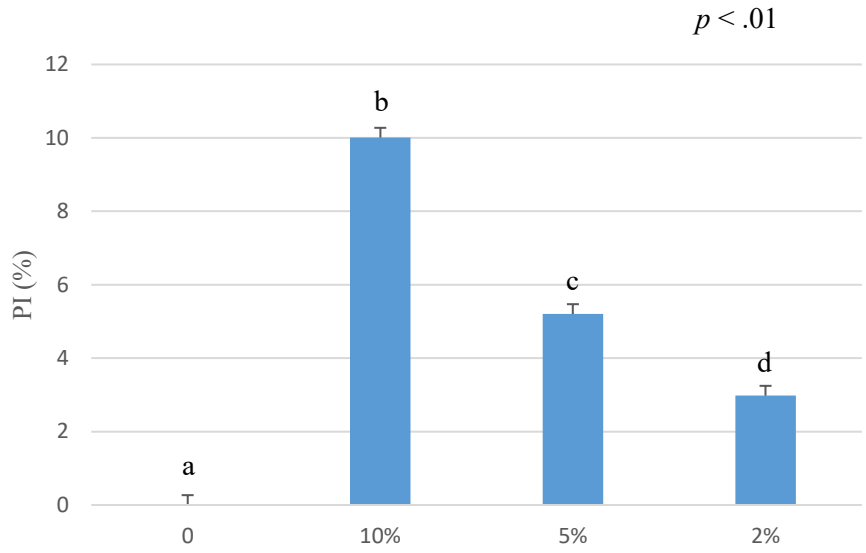

Figure 6: Average of percentage of inhibition (PI%) of *Schizochytrium* spp. ethanol extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *Schizochytrium* spp. ethanol extract 10%, 5%, 2% and blank.
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different (treatment $p < .01$).

The mixture of *A. nodosum* and *Schizochytrium* spp. exhibited an antioxidant capacity of 3.18±0.57 µmol TroloxEq/g for ethanol extract and 10.06±2.73 for water extract (Table 4) (Figure 7, Figure 8). The antioxidant capacity (µmol TroloxEq/g) of the mixture was lower than the antioxidant capacity of *A. nodosum* water extract alone, but the ethanol extracted mixture (3.18±0.57 µmol TroloxEq/g) was comparable to the sum of the antioxidant capacity of both ethanol extracted algae (2.56±0.53 and 0.75±0.31 µmol TroloxEq/g) suggesting that could be possible a synergic effect. Significant differences were observed in a dose-dependent way among the concentration tested expressed as percentage of inhibition (PI%) ($p < .01$).

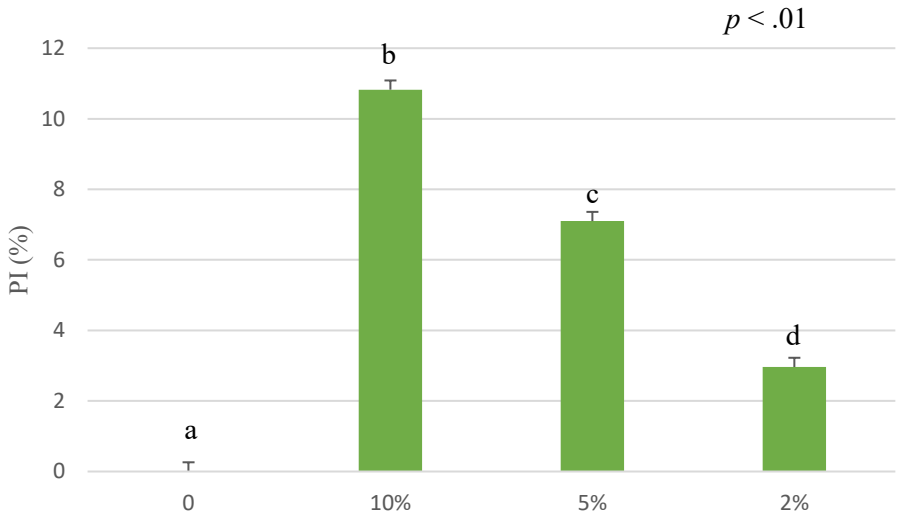

Figure 7: Average of percentage of inhibition (PI%) of *A. nodosum* and *Schizochytrium* spp. mixture (1:1 w/w) ethanol extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* and *Schizochytrium* spp. mixture ethanol extract 10%, 5%, 2% and blank.
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different (treatment $p < .01$).

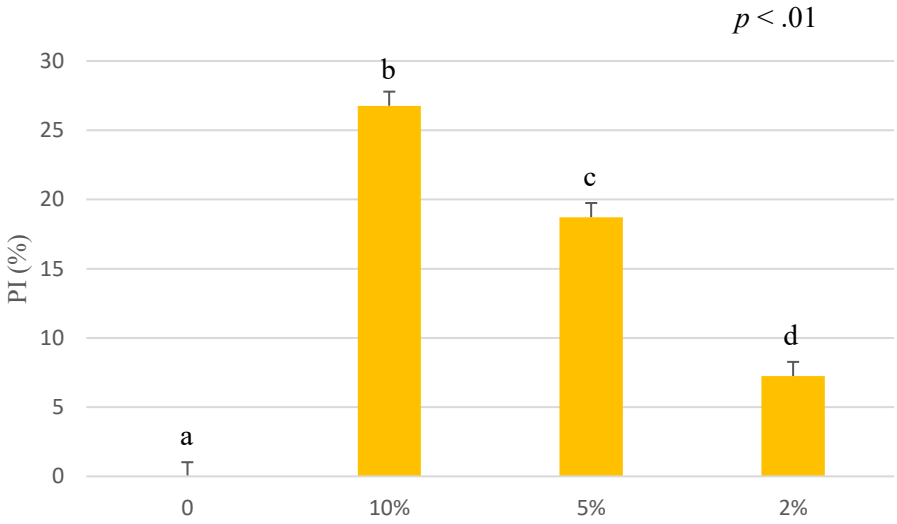

Figure 8: Average of percentage of inhibition (PI%) of *A. nodosum* and *Schizochytrium* spp. mixture (1:1 w/w) water extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* and *Schizochytrium* spp. mixture water extract 10%, 5%, 2% and blank.
Data are shown as least squares means and standard errors.
a-b means (n=3) with different superscripts are significantly different (treatment $p < .01$).

Even if in this study *Schizochytrium* spp. demonstrated lower antioxidant capacity compared to brown algae, several *in vivo* studies have been largely demonstrated the capacity of *Schizochytrium* spp. to improve health status and production efficiency of livestock animals (Lv et al. 2015, Mata et al. 2017). The content of polyphenols, tocopherols and other minor components acts against oxidation (Pandey and Rizvi

2009), in the last decade, interest in the potential health benefits of dietary plant polyphenols as antioxidants has increased rapidly. *Schizochytrium* spp. contains a large amount of DHA that have many beneficial effects. However, high concentrations of n-3 polyunsaturated fatty acids may increase lipid peroxidation and subsequently induce oxidative stress (Vericel et al. 2003). This may explain the lower antioxidant capacity of *Schizochytrium* spp. compared to *Ascophyllum nodosum.*

The antioxidant activity of *A. nodosum* may also be related mainly to phlorotannins, which possess strong antioxidant capacity (Sathya et al. 2017). The antioxidant activity of 55.54±16.05 μmol TroloxEq/g observed for *A. nodosum* is comparable with a strawberry antioxidant capacity (Castrica et al. 2019). Other studies also confirm the antioxidant activity of two polysaccharide groups, laminarin and fucoidans which are both present in brown algae (Kadam et al. 2015). Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities. These activities may be related to their antioxidant activity that consists in the electron-transfer capacity from their molecules to the oxidized radical by scavenging the free radicals (Li et al. 2007).

Considering that *E. coli* diseases are commonly multifactorial, all the stressors during the weaning can reduce the immune defences. Antibacterial and antioxidant compounds could help young animals to maintain the health status, improving defences and thus increasing performance. *A. nodosum* and *Schizochytrium* spp. have displayed suitable characteristics for animal nutrition also revealing interesting bioactivities. The future trends will be the inclusion of algae as ingredient in feed evaluating the optimal level of inclusion related to their bioactivities *in vivo* and define their cost-opportunity.

Conclusions

Considering that algae represent several advantages form agronomic and environmental point of view, they could become one of the valuable sources of food and feed.

A. nodosum and *Schizochytrium* spp. revealed interesting bioactivities *in vitro* and they are promising as future feed additives. Indeed, despite only an antibacterial effect was observed for a limited time, is interesting to notice the synergistic effect supplied from the complementary characteristic of these algal species. *A. nodosum* can modulate the *E. coli* growth, *Schizochytrium* spp. possesses antioxidant capacity and the combination of these two species can enhance the antioxidant power. With the urgent need of

innovative functional feed additives as alternative to antibiotics, these algal species should be considered in animal breeding.

Therefore, more studies on the argument are needed to confirm the *in vitro* obtained results also in livestock conditions. Obtained findings revealed that *A. nodosum* and *Schizochytrium* spp. possess antimicrobial and antioxidant activities, these aspects should be considered as promising for innovative feed additives for functional animal nutrition, since each algal species has displayed different effects that could be used in complementary way.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

AbuGhazaleh AA, Potu RB, Ibrahim S. 2009. The effect of substituting fish oil in dairy cow diets with docosahexaenoic acid-micro algae on milk composition and fatty acids profile. Journal of dairy science. 92(12):6156–9.

Amezcua R, Friendship RM, Dewey CE, Gyles C, Fairbrother JM. 2002. Presentation of postweaning Escherichia coli diarrhea in southern Ontario, prevalence of hemolytic E. coli serogroups involved, and their antimicrobial resistance patterns. Canadian journal of veterinary research. 66(2):73–78.

AOAC. 2005. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Washington, DC.

AOCS. 2009. Approved Procedure Ba 6a-05: Crude fiber analysis in feeds by filter bag technique. In Official Methods and Recommended practices, fourth ed. American Oil Chemists' Society, Champaign, IL.

Bochenski T, Chaturvedi T, Thomsen MH, Schmidt JE. (2019). Evaluation of Marine Synechococcus for an Algal Biorefinery in Arid Regions. Energies. 12(12),2233:1–13.

Cardon ZG, Gray DW, Lewis LA. (2008). The green algal underground: evolutionary secrets of desert cells. Bioscience. 58(2):114–122.

Castrica M, Rebucci R, Giromini C, Tretola M, Cattaneo D, Baldi A. 2019. Total phenolic content and antioxidant capacity of agri-food waste and by-products, Italian Journal of Animal Science. 18(1):336–341.

Combet E, Ma ZF, Cousins F, Thompson B, Lean ME. 2014. Low-level seaweed supplementation improves iodine status in iodine-insufficient women. British Journal of Nutrition. 112(5):753–761.

Dhama K, Chakraborty S, Kapoor S, Tiwari R, Kumar A, Deb R, Rajagunalan S, Singh R,Vora K, Natesan S. 2013. One world, one health-veterinary perspectives. Advances in Animal and Veterinary Sciences. 1(1):5–13.

Dierick N, Ovyn A, De Smet S. 2010. *In vitro* assessment of the effect of intact marine brown macro-algae *Ascophyllum nodosum* on the gut flora of piglets. Livestock science. 133(1–3):154–156.

Directive 2002/32/EC of the European parliament and of the council of 7 May 2002 on undesirable substances in animal feed.

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC). 2013. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2011. EFSA Journal. 11:3196–3359.

European Food Safety Authority (EFSA). 2012. Guidance on Risk Assessment for Animal Welfare. EFSA Panel on Animal Health and Welfare (AHAW).

Evans FD, Critchley AT. 2014. Seaweeds for animal production use. Journal of applied phycology. 26(2):891–899.

Fievez V, Boeckaert C, Vlaeminck B, Mestdagh J, Demeyer D. 2007. *In vitro* examination of DHA-edible micro-algae: 2. Effect on rumen methane production and apparent degradability of hay. Animal feed science and technology. 136(1–2):80–95.

Gardiner GE, Campbell AJ, O'Doherty JV, Pierce E, Lynch PB, Leonard FC, Stantona C, Rossa RP, Lawlor PG. 2007. Effect of *Ascophyllum nodosum* extract on growth performance, nutrient digestibility, carcass characteristics and selected intestinal microflora populations of grower-finisher pigs. Animal Feed Science and Technology. 141(3–4):259–273.

Gouveia L, Oliveira AC. 2009. Microalgae as a raw material for biofuels production. J Ind Microbiol Biotechnol. 36:269–74.

Hejna M, Gottardo D, Baldi A, Dell'Orto V, Cheli F, Zaninelli M, Rossi L. 2018. Nutritional ecology of heavy metals. Animal. 12(10):2156–2170.

Jiang Y, Fan K-W, Wong RT-Y, Chen F. 2004. Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*. Journal of Agricultural and Food Chemistry. 52:1196–2000.

Jiménez JT, O'Connell S, Lyons H, Bradley B, Hall M. 2010. Antioxidant, antimicrobial, and tyrosinase inhibition activities of acetone extract of *Ascophyllum nodosum*. Chemical Papers. 64(4):434–442.

Kadam SU, Tiwari BK, O'donnell CP. 2015. Extraction, structure and biofunctional activities of laminarin from brown algae. International Journal of Food Science & Technology. 50(1):24–31.

Kim HJ, Dasagrandhi C, Kim SH, Kim BG, Eom SH, Kim YM. 2018. *In vitro* antibacterial activity of phlorotannins from edible brown algae, *Eisenia bicyclis* against streptomycin-resistant *Listeria monocytogenes*. Indian journal of microbiology. 58(1):105–108.

Li HB, Cheng KW, Wong CC, Fan KW, Chen F, Jiang Y. 2007. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food chemistry. 102(3):771–776.

Lv J, Yang X, Ma H, Hu X, Wei Y, Zhou W, Li L. 2015. The oxidative stability of microalgae oil (*Schizochytrium aggregatum*) and its antioxidant activity after simulated gastrointestinal digestion: Relationship with constituents. European journal of lipid science and technology. 117(12):1928–1939.

Machu L, Misurcova L, Vavra Ambrozova J, Orsavova J, Mlcek J, Sochor J, Jurikova T. 2015. Phenolic content and antioxidant capacity in algal food products. Molecules. 20(1):1118–1133.

Madeira MS, Cardoso C, Lopes PA, Coelho D, Afonso C, Bandarra NM, Prates JA. 2017. Microalgae as feed ingredients for livestock production and meat quality: A review. Livestock Science. 205:111–121.

Makkar HP, Tran G, Heuzé V, Giger-Reverdin S, Lessire M, Lebas F, Ankers P. 2016. Seaweeds for livestock diets: a review. Animal Feed Science and Technology. 212:1–17.

Mata TM, Martins AA, Caetano NS. 2010. Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews. 14(1):217–232.

Munir N, Sharif N, Naz S, Manzoor F. 2013. Algae: a potent antioxidant source. Sky Journal of Microbiology Research. 1(3):22–31.

Murphy D, Ricci A, Auce Z, Beechinor JG, Bergendahl H, Breathnach R. et al. 2017. EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). EFSA Journal. 15(1):1–245.

Myers JA, Curtis BS, Curtis WR. 2013. Improving accuracy of cell and chromophore concentration measurements using optical density. BMC biophysics. 6(1):1–15.

Pandey KB, Rizvi SI. 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative medicine and cellular longevity. 2(5):270–278.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology and medicine. 26(9–10):1231–1237.

Regulation (EC) No 1831/2003 of the European parliament and of the council of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance).

Rossi L, Di Giancamillo A, Reggi S, Domeneghini C, Baldi A, Sala V, Dell'Orto V, Coddens A, Cox E, Fogher C. 2013. Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. Journal of veterinary science. 14(3):263–270.

Rossi L, Pinotti L, Agazzi A, Dell'Orto V, Baldi A. 2014. Plant bioreactors for the antigenic hook-associated flgK protein expression. Italian Journal of Animal Science. 13(1):23–29.

SAS Institute. 2009. SAS/STAT® 9.4 User's Guide. 2nd ed. SAS Institute Inc., Cary, NC.

Sathya R, Kanaga N, Sankar P, Jeeva S. 2017. Antioxidant properties of phlorotannins from brown seaweed *Cystoseira trinodis* (Forsskål) C. Agardh. Arabian Journal of Chemistry. 10:S2608–S2614.

Verdonck F, Cox E, van Gog K, Van der Stede Y, Duchateau L, Deprez P, Goddeeris BM. 2002. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic Escherichia coli strain or an F18 verotoxigenic Escherichia coli strain. Vaccine. 20(23–24):2995–3004.

Wang Y, Xu Z, Bach SJ, McAllister TA. 2009. Sensitivity of Escherichia coli to seaweed (*Ascophyllum nodosum*) phlorotannins and terrestrial tannins. Asian-Australasian Journal of Animal Sciences. 22(2):238–245.

Windisch W, Schedle K, Plitzner C, Kroismayr A. 2008. Use of phytogenic products as feed additives for swine and poultry. Journal of animal science. 86(14):E140–E148.

Zoetendal EG, Smith AH, Sundset MA, Mackie RI. 2008. The BaeSR two-component regulatory system mediates resistance to condensed tannins in *Escherichia coli*. Applied and Environmental Microbiology. 74(2):535–539.

animals

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Article

In Vitro Digestion of Chestnut and Quebracho Tannin Extracts: Antimicrobial Effect, Antioxidant Capacity and Cytomodulatory Activity in Swine Intestinal IPEC-J2 Cells

Serena Reggi †, Carlotta Giromini *,†, Matteo Dell'Anno, Antonella Baldi, Raffaella Rebucci and Luciana Rossi

Brief Introduction to the Study:
This study demonstrated the antimicrobial activity, antioxidant effect and simulated the gastrointestinal digestibility of chestnut and quebracho tannins as functional feed additives for animal feed. The *in vitro* study allowed to evaluate functional properties of tannins from different sources and predict their bioaccessibility *in vivo*. The importance of the study relied on the fact that this preliminary evaluation allowed to highlight that the combination of chestnut and quebracho tannins could maximise the antimicrobial activity compared to single chestnut or quebracho tannins alone, probably due to different classes of polyphenols present in the tannin extracts. In addition, in this manuscript tested multiple concentrations of tannins in order to optimize their further dietary supplementation *in vivo*. This study played a key role to settle the optimal conditions, without the use of animals for experimental purposes, prior to testing the effect of tannins in post-weaning piglets.

3.2 *In Vitro* Digestion of Chestnut and Quebracho Tannin Extracts: Antimicrobial Effect, Antioxidant Capacity

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Simple Summary: *Castanea sativa* Mill. (Fagaceae) is the predominant sweet chestnut tree in Europe. Despite the significant economic value of chestnuts as sources of food and wood, the high content of tannin also increases the value of sweet chestnut trees.

Quebracho trees (*Schinopsis* spp., family *Anacardiaceae*) grow mainly in Argentina and Paraguay. Quebracho extract obtained from *Schinopsis* spp. contains 15% to 21% pure tannin. Tannins extracted from these plants have been applied in intensive swine farms due to their ability to improve animal performance and health. However, there are contrasting results regarding the bio-accessibility of chestnut and quebracho and their relative antioxidant activity and growth-rate reducing ability on *E. coli*, which ultimately affect their benefits in terms of intestinal health and animal production. Our results demonstrate that chestnut and quebracho exert a growth inhibitory activity against Enterotoxigenic *E. coli* (ETEC) species and antioxidant capacity directly, without extraction and after *in vitro* digestion. Our findings not only suggest that the combined use of chestnut and quebracho can maximize their functional effects, but also that an appropriate dosage of tannins may be key in terms of their effect on bacteria and cells.

Abstract: Quebracho (Qu) and chestnut (Ch) are natural sources of tannins and they are currently used in animal nutrition as feed ingredients. However, to date the bio-accessibility, antimicrobial, antioxidant, and intestinal epithelial cell stimulatory doses of Qu and Ch have not been determined. Our study investigates the antioxidant and *E. coli* F4+ and F18+ growth inhibitory activity of Qu, Ch, and their combinations after solubilization in water (to evaluate the already bio-accessible molecules) and after simulated gastro-intestinal digestion *in vitro*. The effect of an *in vitro* digested Ch and Qu combination was also tested on intestinal epithelial IPEC-J2 cells experimentally stressed with hydrogen peroxide (H_2O_2) and Dextran Sodium Sulphate (DSS). The results showed that undigested Qu and Ch alone, and in combination, exerted a valuable antioxidant capacity and *E. coli* F4+ and F18+ growth inhibitory activity. The concentration of 1200 μg/mL exhibited the highest *E. coli* growth inhibitory activity for all the samples tested. In addition, after in vitro digestion, Qu and Qu50%–Ch50% maintained *E. coli* growth inhibitory activity and a modest antioxidant capacity. Three hours pre-treatment with *in vitro* digested Qu50%–Ch50% counteracted the H2O2 and DSS experimentally-induced stress in the intestinal IPEC-J2 cells. Ch and Qu tannin extracts, particularly when combined, may exert E. coli F4+ and F18+ growth inhibitory activity and valuable antioxidant and cell viability modulation activities.

Keywords: plant extracts; tannin; *in vitro* digestion; growth inhibition; enterotoxigenic *E. coli*; antimicrobial activity; antioxidant capacity; IPEC-J2 intestinal cell

1. Introduction

Plant tannins are water-soluble polyphenolic compounds of a variable molecular weight, which are abundant in nature [1]. They can be classified into condensed (molecular weight: 1000–20,000) and hydrolysable groups (molecular weight: 500–3000) [2,3]. They have different nutritional significance and adverse effects. Chestnut (Ch, *Castanea sativa* Mill.) and quebracho (Qu, *Schinopsis* spp.) tannin extracts have been used for over ten years in animal feeding [4–6]. Although tannins can interfere with the digestion of nutrients as they bind proteins and delay the absorption of sugar and lipids, several studies have reported that the addition of Qu and Ch to animal feed improved the growth performance and health in both ruminants and monogastric animals [4,5,7].

Although Ch and Qu tannins have been studied in both weaned and fattening piglets in terms of their antimicrobial activities [8,9], there is still no consensus on the appropriate Ch and Qu tannin dose that maximizes the beneficial effect and minimizes the anti-nutritional value of tannins. Moreover, even though Qu and Ch have shown significant biological properties *in vivo*, little is known about the bio-accessibility and bioavailability after digestion and the biological effects of such compounds used alone and in combination. Due to their chemical composition, they can exert antimicrobial, antiviral, antioxidant, and antimutagenic effects locally in the intestine as unabsorbable complex structures [10]. In this scenario, *in vitro* digestion models for nutrient evaluation are important for studying both the physiology of certain segments of the digestive tract and the digestive and bioactive characteristics of tannins. In pig livestock, Enterotoxigenic *E. coli* (ETEC) is the most important pathotype causing both neonatal and post-weaning diarrhoea (PWD), which are responsible for significant economic losses worldwide and are the most common reason for the prescription of antimicrobials in intensive systems [11,12]. The proliferation of ETEC strains in the gut and their pathogenicity can be influenced by the expression of fimbrial adhesins, which bind to specific receptor sites on small intestinal enterocytes enabling the bacteria to colonize the small intestine [13,14]. ETEC strains equipped with F4 and F18 adhesive fimbriae show a high virulence and are the most common serotypes isolated in animals elected by PWD [15]. As an alternative to antibiotics, new compounds are urgently needed to control enteric diseases and PWD in pig livestock [14]. Tannins are suitable due to their valuable extra-nutritional properties. However, interactions with bacterial toxins seem to be specific, as only a few tannins are able to reduce ETEC diarrhoea [16].

In the present study, we evaluated the *in vitro* antioxidant and *E. coli* F4+ and F18+ growth inhibitory activities of Qu, Ch, and their combinations in two

experimental conditions: (i) After solubilization in water, to demonstrate the direct effect of bioactive compounds responsible for bacterial growth inhibition and antioxidant activities; (ii) after *in vitro* gastro-intestinal digestion to evaluate the bio-accessibility of bioactive molecules responsible for such activities. We also tested the ability of the Qu–Ch mixture to counteract H_2O_2 and DSS-induced stress in IPEC-J2 as a cell model of the intestinal swine epithelium.

2. Materials and Methods

2.1 Chemical Analysis of Chestnut and Quebracho Tannin and Sample Preparation

Ch and Qu extracts tested in the present study were obtained by hot water solubilization and contain 75 g of tannin/100 g of dry matter (Silvateam S.p.A, San Michele Mondovi, Italy).

They were tested in terms of their chemical composition (AOAC, 2005; EU regulation 152/2009) and the data are included in Supplementary Table S1.

The chemical composition of the Ch and Qu was analyzed in the laboratory for oven-dried samples (65 °C) to determine the moisture, and then ground through a 1-mm screen. Ash, crude protein (CP), neutral detergent fiber (NDF), and ether extract (EE) were determined following the methods of the Association of Offcial Analytical Chemists (AOAC, 2005) (Table 1) [17].

Table 1. Chemical composition of Qu and Ch tannins.

% on Dry Matter	Moisture EU Regulation 152/2009	Ash AOAC 942.05 (2005)	Neutral Detergent Fiber (NDF) AOAC 2002.04 (2005)	Crude Protein AOAC 2001.11 (2005)	Ether Extract AOAC 2001.11 (2005)
Qu	4.82 ± 0.04	2.01 ± 0.15	<0.5	1.40 ± 0.02	<0.5
Ch	5.30 ± 0.04	0.99 ± 0.05	<0.5	0.90 ± 0.1	<0.5

Ch (Ch100%), Qu (Qu100%), and three different mixtures of Ch and Qu tannins (Qu75%–Ch25%, Qu50%–Ch50% and Qu25%–Ch75%) were dissolved in hot water (100 mg/mL), neutralized (pH 7), and filter sterilized (0.22 μm filter, Millipore).

In both tannin extracts, the crude protein content was below 1.5% on a DM basis, and the fat and NDF concentrations were negligible.

Tannin mixtures were obtained by mixing the Ch100% and Qu100% powders to give the following:

Qu:Ch ratios:

1:1 (Qu50%–Ch50%);

1:3 (Qu25%–Ch75%);

3:1 (Qu75%–Ch25%).

2.2 Total Antioxidant Capacity – ABTS Assay

Antioxidant capacity (AOX) was determined in Ch100%, Qu100%, Qu75%–Ch25%, Qu50%–Ch50% and Qu25%–Ch75% samples (100mg/mL) following Re et al. (1999) [18] with modifications. Trolox stock solution (2.5 mM in distilled water) was used to produce the standard curve. A solution of 2,20-azinobis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) (7 mM) was prepared with potassium persulfate (140 mM) in distilled water and left to react in the dark for 12–16 h to produce the ABTS•+ solution. For the study of AOX capacity, the ABTS•+ solution was diluted with phosphate phosphate-buffered saline, pH 7.4, (PBS) to obtain an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. A volume of 20 μL of the sample or Trolox standard was mixed with 2 mL of ABTS•+ working solution and incubated in the dark for 6 min at room temperature before measuring absorbance at 734 nm on a spectrophotometer (V-630 UV-VIS Spectrophotometer, JASCO, Germany). Solvent blanks were included in each assay. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of Trolox standard curve. AOX results were expressed as μmol Trolox equivalents (TE)/g extract.

2.3 E. coli Growth Inhibitory Activity

The *E. coli* F4+ and F18+ growth inhibitory activity of Ch100%, Qu100% and Qu75%–Ch25%, Qu50%–Ch50% and Qu25%–Ch75% was evaluated for *in vitro* cultures of *E. coli* F4+ and F18+. Two ETEC strains, harbouring F4 (F4+) and F18 (F18+) adhesive fimbriae respectively, were obtained from IZSLER (Brescia, Italy). The bacteria were grown at 37 °C with shaking (150 rpm) in LB broth for 12 h prior to being used as inoculants for all experiments. F4+ and F18+ were characterized by PCR in terms of their virulence factors F4 and F18 (see the Supplementary Materials).

Overnight-grown E. coli F4+ and F18+ were inoculated in tubes containing 15 mL of LB medium supplemented with 0, 200, 400, 600, 800, and 1200 μg/mL of each tannin. Prior to inoculation, the bacterial cultures were adjusted to identical densities by spectrophotometry (600 nm) across the two strains. All tubes were incubated aerobically with shaking (150 rpm) at 37 °C.

The bacterial growth was determined via measurement of the optical density of each culture at 600 nm (OD600) at 60-min intervals in a spectrophotometer (V-630 UV-VIS Spectrophotometer, JASCO, Germany). Bacterial-free tubes with equivalent concentrations of tannins were used as blanks to subtract the background turbidity caused by tannin-protein interactions [19].

All data obtained from the optical density evaluation were converted to log-transformed based cell count (CFU/mL) by a calibration curve, obtained by monitoring

the *E. coli* F4+ and F18+ growth over time, in the same experimental conditions, using the classic plate counting method (data not shown) [20].

2.4. Determination of Minimal Inhibitory Concentration (MICs) and Minimal Bactericidal Concentration (MBC)

Minimum inhibitory concentrations were determined in 96-well microplates by preparing a gradient of tannin solutions (ranging from 10 mg/mL to 0 mg/mL). Briefly, 100 µL of the tannin solutions, 100 µL of LB broth and 10 µL of an *E. coli* culture (approximately 106 CFU/mL) were inoculated in each well of the plate, except for the blank wells, and incubated at 37 °C for 18 h. Bacterial growth was determined by the change in absorbance after reading the microplates at 600 nm in a spectrophotometer reader (BioRAD). The MIC was defined as the lowest tannin concentration that did not produce turbidity by comparison with tannin-free control (0 mg/mL) [21]. The experiment was repeated three times and the results were expressed as average values.

In vitro bactericidal analyses were conducted with 0, 6, 7, 8, 9, and 10 mg/mL of Ch100%, Qu100%, Qu75%–Ch25%, Qu50%–Ch50%, and Qu25%–Ch75% at 24 h incubation in LB medium. Samples taken from all cultures were serially diluted (10-fold increments) in a sterile physiological solution. Dilutions were plated on LB agar and incubated overnight at 37 °C. Colonies grown on agar plates were directly counted after 24 h of incubation. The percentage bactericidal effect was calculated from the control vs. mg of tannins per mL. The lowest tannin concentration that did not yield any colony growth after 24-h incubation was designed as the minimum bactericidal concentration (MBC) [22].

2.5. Chestnut and Quebracho Tannins In Vitro Digestion and Calculation of Digestibility

Based on antimicrobial and antioxidant results obtained in tannin water extracts, we investigated the E. coli growth inhibitory and antioxidant activities of Ch100%, Qu100%, and Qu50%–Ch50% after *in vitro* digestion. The digestion was performed according to the method set up and validated by Minekus et al. [23], and further adapted by our group [24,25].

Briefly, 20 g of each tannin powder (Ch100%, Qu100%, and Qu50%–Ch50%) was mixed with 150 mL of distilled H_2O and kept on an orbital shaker at 150 rpm for 5 min. The digestion procedure involved three phases. For the oral phase, 6.66 mg α-amylase in 2.1 mL of 1 mM $CaCl_2$, pH 7 was added to the samples which were then incubated for 30 min at 37 °C on a shaker. For the gastric phase, the pH was decreased to 2 with 6 M HCl and 0.9 g of pepsin was added in 8.3 mL of 0.1 M HCl. The samples were then incubated for 120 min at 37 °C on a shaker. For the small intestinal phase, the pH was

increased to 7 with 6 M NaOH and 0.2 mg pancreatin and 1.2 g bile in NaHCO3 0.5 M were added to the samples before carrying out the final incubation of 180 min at 37 °C on a shaker.

A blank sample (enzymes of the digestion alone), along with a positive and negative control were included as reference samples in all the digestions performed (n = 3).

At the end of digestion, the total digesta obtained was transferred to a 3-kDa cut-off membrane (Vivaspin 20, Sartorius, Göttingen, Germany). Each filter was previously activated with 0.1% BSA solution. Samples were centrifuged for 20 min at 3500× g (5 °C). Aliquots from the filtrate were sampled and snap-frozen in liquid nitrogen to stop enzyme activity, before storing at -80 °C for further experiments.

The undigested fraction was collected and used to calculate *in vitro* digestibility as detailed by Castrica et al. [24].

2.6. Antioxidant and E. coli Growth Inhibitory Activities of In Vitro Digested Tannins

Antioxidant and *E. coli* F4+ and F18+ growth inhibitory activity activities of physiological extracts of Ch100%, Qu100%, and Qu50%–Ch50% were performed as described above.

2.7. Effects of In Vitro Digested Chestnut and Quebracho Tannins on Intestinal IPEC-J2 Cell Viability

IPEC-J2 cells are intestinal porcine enterocytes isolated from the jejunum of a neonatal unsuckled piglet (ACC 701, DSMZ, Braunschweig, Germany). The IPEC-J2 cell line is unique as it is derived from the small intestine and is not transformed nor tumorigenic in nature [26]. The IPEC-J2 cells were cultured in DMEM/F-12 mix (Dulbecco's Modified Eagle Medium, Ham's F-12 mixture) supplemented with HEPES, fetal bovine serum (FBS), penicillin/streptomycin and cultivated in a humid chamber at 37 °C with 5% CO2. All experiments were performed using IPEC-J2 cells within six cell passages (passages 16 to 22) to ensure reproducibility.

IPEC-J2 cells were seeded at a density of 1.5–2 × 10^5 cells/mL in 96-well plates and cultured for 24 h. In addition, dose-response curves (cell viability) of *in vitro* digested Ch, Qu, and Qu50%–Ch50% were performed on IPEC-J2 cells based on preliminary experiments on bacteria (0–1200 μg/mL) obtained in previous experiments. Cell viability was determined after three hours of tannin treatment by a colorimetric proliferation assay (MTT test) in accordance with the manufacturer's instructions.

In a second set of experiments, IPEC-J2 cells at sub-confluence were pre-treated with Ch, Qu, and Qu50%–Ch50% for three hours, and further challenged with H_2O_2 or

with DSS to induce chemical stress in the cell culture. Hydrogen peroxide was applied at a concentration of 0.5 mM for 1-h incubation. DSS at a concentration of 2% was applied for 24 h. Time and doses of H_2O_2 incubation were based on preliminary data and on the literature [27]. Time and doses of DSS incubation were based on our preliminary study, where IC50 was calculated for IPEC-J2 cells [28].

2.8. Statistical Analysis

Statistical analysis was performed using GraphPad-Prism 8. *E. coli* growth data (OD600) were $\log_{10}$ transformed prior to statistical analysis. *E. coli* growth data were subjected to analysis of variance using the MIXED procedure. The model included the fixed effects of treatments (Ch100%, Qu100%, Qu75%–Ch25%, Qu50%–Ch50%, and Qu25%–Ch75%), time and time x treatment. One-way ANOVA was used to analyse antioxidant and cell viability data. The differences between means were compared using Tukey's test and considered statistically significant at $p < 0.05$. Data are presented as least square means ± SEM.

3. Results

3.1. Antioxidant and E. coli F4+ and F18+ Growth Inhibitory Activity of Chestnut and Quebracho Tannin Water Extracts

3.1.1. Total Antioxidant Capacity—ABTS Assay

Chestnut and Qu tannin extracts showed an antioxidant (AOX) capacity. Among the tested samples, Qu25%–Ch75% showed the highest AOX capacity (6860 ± 121.9 μmol TE/g tannin powder). Ch100% exhibited higher AOX compared with Qu100% (5243.33 ± 113.1 vs. 3164.81 ± 166.2 μmol TE/g), which showed the lowest AOX capacity compared with all the other samples tested (Figure 1). The AOX capacity of Ch100% and Qu50%–Ch50% was comparable ($p > 0.05$). Trolox at a concentration of 2000 μM was included as an internal control and showed an AOX capacity of 1.828 μmol TE/g.

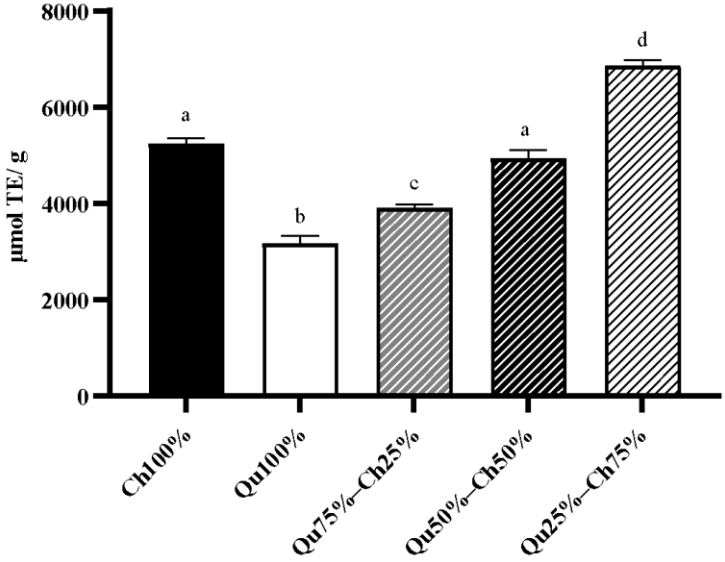

Figure 1. Ch75% (100 mg/mL). Data are presented as lsmeans ± SEM (n = 3). The different superscript letters indicate a significant difference at $p < 0.05$ (one-way ANOVA). 2000 µM Trolox was included as an internal control.

3.1.2. *E. coli* Growth Inhibitory Activity

The growth of *E. coli* F4+ and F18+ strains was tested in the absence or presence of different concentrations (0–1200 µg/mL) of Ch and Qu tannin extracts (see Supplementary Figure S2). In general, a dose-dependent effect was observed at each time point for both Ch and Qu treatments, for which, the maximum growth inhibition was observed at a concentration of 1200 µg/mL. Based on this result and on previous unpublished data on the synergistic effect of tannin extracts, we therefore compared the effects of Qu, Ch and of different combinations of Ch and Qu (Qu75%–Ch25%, Qu50%–Ch50%, and Qu25%–Ch75%) at a concentration of 1200 µg/mL (Figure 2) in *E. coli* F4+ and F18+. Generally, in both F4+ and F18+ the combined use of Ch and Qu showed a synergistic activity in the inhibition of F4+ and F18+ growth. Overall, the combinations with the highest Ch concentration (Qu50%–Ch50% and Qu25%–Ch75%) were the most effective (Figure 2).

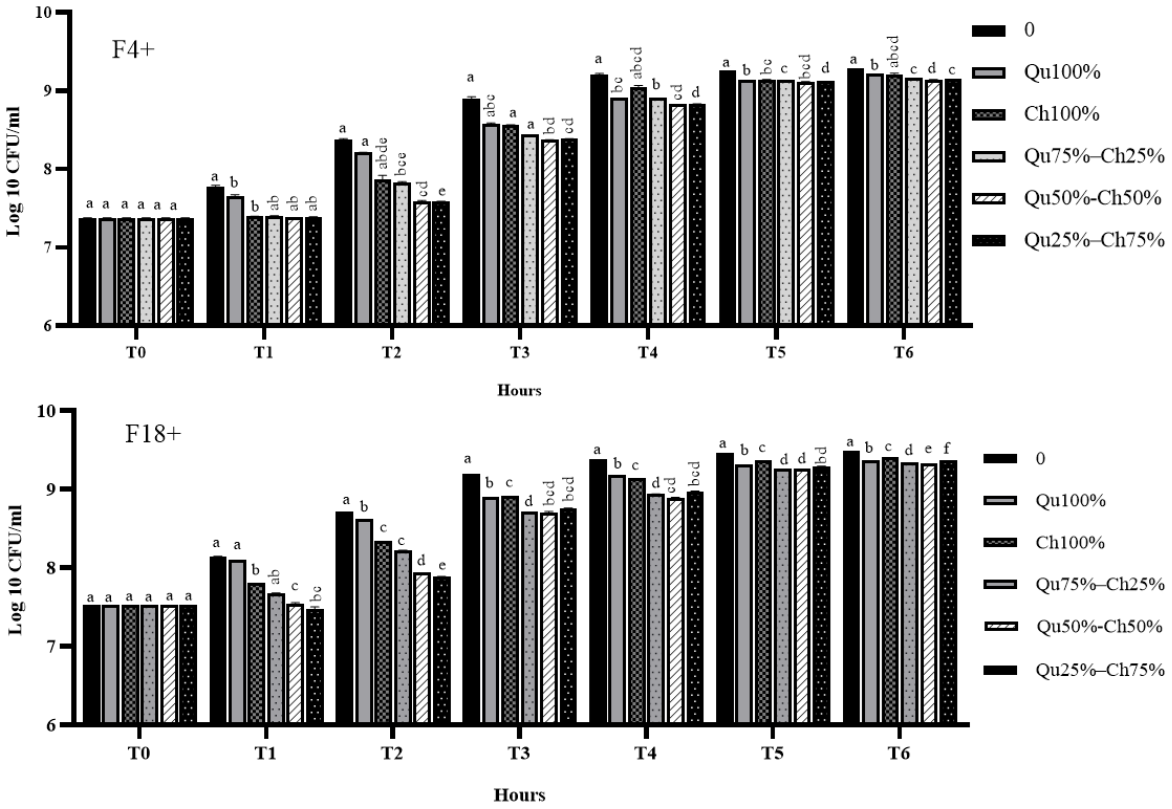

Figure 2. Effects of 1200 μg/mL of Qu (Qu100%), Ch (Ch100%), Qu75%–Ch25%, Qu50%–Ch50%, and Qu25%–Ch75% on E. coli F4+ and F18+ growth over time (T). Data are expressed as log10 CFU/mL lsmeans ± S.E.M. (n = 3, mixed ANOVA). Different superscript letters indicate significant differences at $p < 0.05$ among different concentrations within the same time point.

3.1.3. Determination of Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentration (MBC)

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of Qu and Ch tannins and of their combinations were calculated in E. coli F4+ and F18+ (Table 2). Data showed a similar bactericidal activity of both Ch and Qu, in particular in tannin combinations. The bactericidal effects of 6 mg/mL of Qu and Ch tannins was partial (55–62% for F4+ and 50–60% for F18+) after 24 h of incubation. In contrast, there was a complete bactericidal effect when Ch and Qu were evaluated, after 24 h of incubation, at a concentration of 9 mg/mL for E. coli F4+ and 8 mg/mL for E. coli F18+ (Table 2).

Table 2. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of Qu and chestnut tannins on E. coli F4+ and F18+.

Bacteria	MIC (mg/mL)					MBC (mg/mL)				
	Qu100%	Ch100%	Qu75%-Ch25%	Qu50%-Ch50%	Qu25%-Ch75%	Qu100%	Ch100%	Qu75%-Ch25%	Qu50%-Ch50%	Qu25%-Ch75%
E. coli F4+	6	7	6	6	6	9	9	8	8	8
E. coli F18+	6	7	6	6	6	8	8	8	8	8

3.2. Antioxidant and E. coli Growth Inhibitory Activities of In Vitro Digested Chestnut and Quebracho Tannin

Qu100%, Ch100%, and Qu50%-Ch50% were *in vitro* digested and subsequently tested for their antioxidant, *E. coli* growth and cytomodulatory activity in IPEC-J2 cells. The combination Qu50%-Ch50% was also tested on IPEC-J2 cells subjected to stress chemically induced by H2O2 and DSS. The *in vitro* digestion enabled the digestibility values to be calculated for Ch and Qu, which corresponded to 66.16% (of DM) and 71.93% (of DM) for Ch and Qu, respectively.

3.2.1. Total Antioxidant Capacity – ABTS Assay

Physiological extracts of Ch and Qu tannins showed 2433.33 ± 114. 15 and 1944. 81 ± 151.95 μmol TE/g. Physiological extracts of Qu50%–Ch50% showed an AOX of 2434.76 ± 211.80 μmol TE/g (Figure 3).

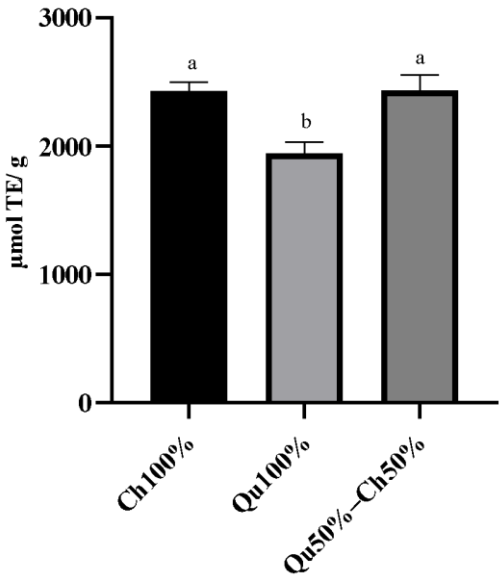

Figure 3. Antioxidant capacity of *in vitro* digested Ch100%, Qu100% and Qu50%–Ch50%. Data are presented as lsmeans ± SEM (n = 3). The different superscript letters indicate a significant difference at $p < 0.05$ (one-way ANOVA). 2000 μM Trolox was included as an internal control.

3.2.2. *E. coli* Growth Inhibitory Activity

The activity of Ch, Qu, and Qu50%–Ch50% on E. coli growth was further tested after *in vitro* digestion (Figure 4).

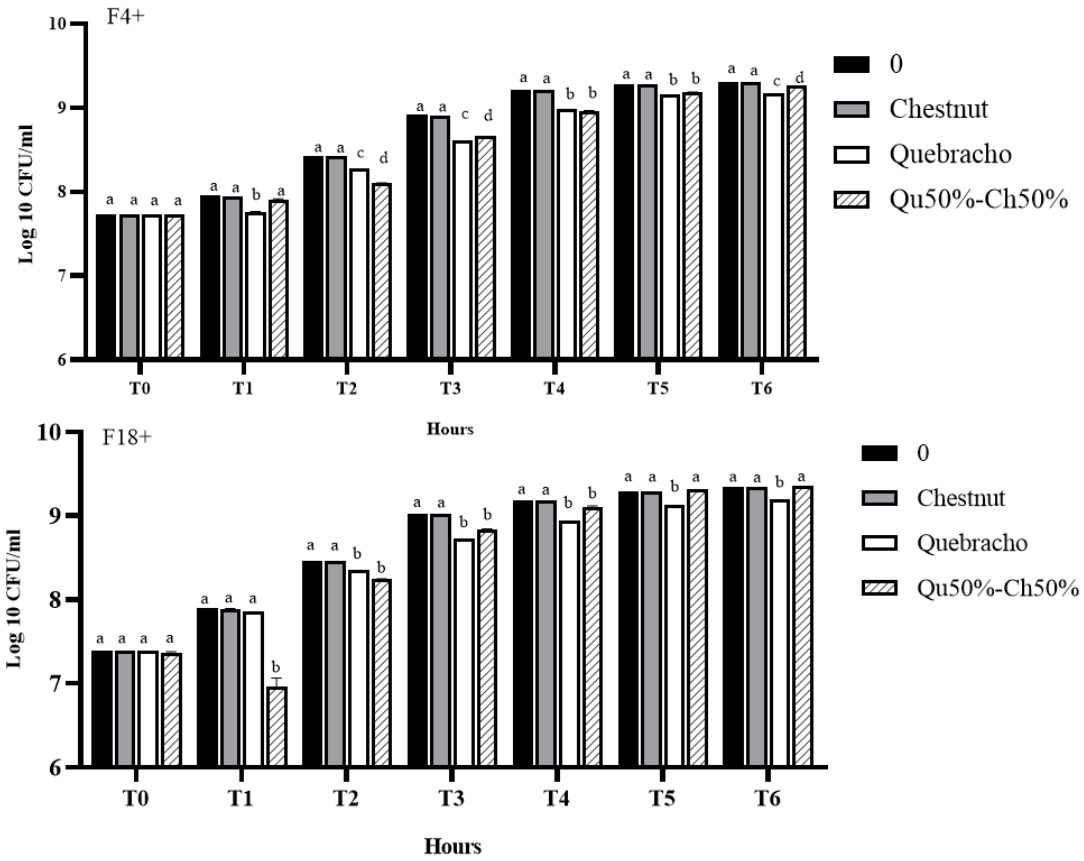

Figure 4. Effects of 1200 μg/mL of Ch 100%, Qu 100%, and Qu50%–Ch50% after *in vitro* digestion on *E. coli* F4+ and F18+ growth over time. Data are expressed as log10 CFU/mL lsmeans ± S.E.M. (n = 3, mixed ANOVA). Different superscript letters indicate significant differences at $p < 0.05$ among different concentrations within the same timepoint.

The *E. coli* growth inhibitory activity of Qu on was significantly higher compared to all other treatments from T1 to T6 ($p < 0.05$) for F4+, but was significantly higher compared to all other treatments from T2 to T6 ($p < 0.05$) for F18+. Qu50%–Ch50% activity was significantly higher from T2 to T6 for F4+ compared with the control, and was significantly higher ($p < 0.05$) from T1 to T4 for F18+. Ch, however, did not show a significant E. coli growth inhibitory activity after *in vitro* digestion.

3.2.3. Effect of *In Vitro* Digested Chestnut and Quebracho Tannin Extracts on IPEC-J2 Cells Chemically Challenged with H_2O_2 and DSS

The *in vitro* digested Qu, Ch, and Qu50%-Ch50% were also tested for swine intestinal epithelial IPEC-J2 to determine whether tannins also affect the viability of the cells. Dose-response curves with several concentration of *in vitro* digested Qu, Ch, and Qu50%-Ch50% tannins were tested on IPEC-J2 cells and the viability was assessed after three hours of incubation (Figure 5).

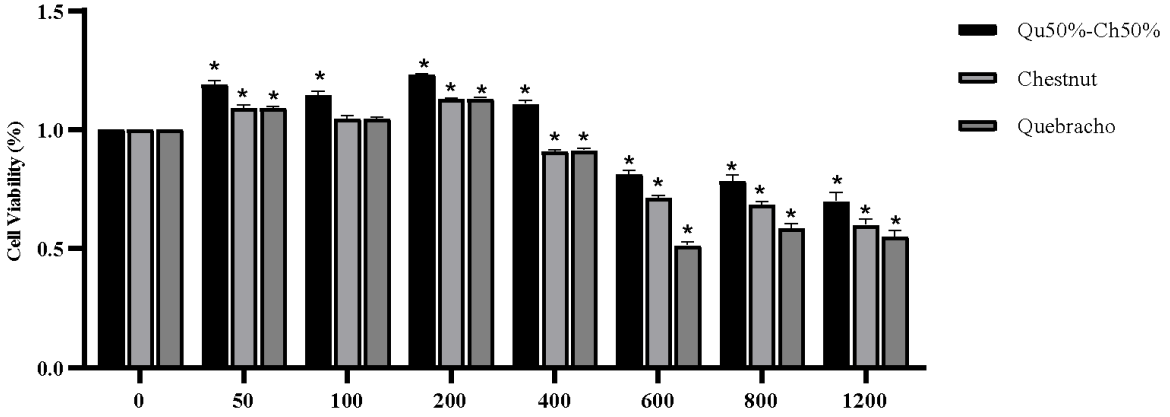

Figure 5. Effect of different concentrations (0-1200 μg/mL) of *in vitro* digested Qu50%-Ch50% on IPEC-J2 cell metabolic activity (expressed as cell viability, MTT assay). Data are expressed as lsmeans ± SEM (n = 3, one-way ANOVA). * indicates significant differences at $p < 0.05$ compared to respective control wells (0 μg/mL).

The results showed that at the highest concentrations of *in vitro* digested Ch, Qu and Qu50%-Ch50% tested (1200-600 μg/mL), IPEC-J2 cell viability was significantly reduced compared with 0 μg/mL; at a concentration of 400 μg/m, Qu50%-Ch50% significantly increased IPEC-J2 cell viability, Ch and Qu reduced cell viability ($p < 0.05$). At the lowest concentrations tested (200-50 μg/mL), IPEC-J2 cell viability was unaltered or increased by Ch and Qu treatment and was significantly increased by Qu50%-Ch50% treatment (Figure 5). Qu50%-Ch50% was the most effective treatment and stimulated IPEC-J2 cell viability after three hours of incubation. Based on the trophic effect observed in IPEC-J2 cells treated with *in vitro* digested Qu50%-Ch50%, this combination was chose for the cell-challenging experiments.

We also tested the trophic effect of Qu50%-Ch50% on IPEC-J2 cells previously stressed with H_2O_2 or DSS. In particular, IPEC-J2 cells were pre-treated for three hours with *in vitro* digested Qu50%-Ch50% at different concentrations (50-1200 μg/mL) and further challenged with H_2O_2 for 1 h or with DSS for 24 h, to stimulate *in vitro* conditions of oxidative and inflammatory stress at the level of intestinal cell epithelium.

In the DSS-challenged IPEC-J2 cells (Figure 6a), the lowest concentrations of Qu50%-Ch50% (50-400 μg/mL) significantly counteracted DSS-induced stress by increasing cell viability. However, at concentrations from 600-1200 μg/mL Qu50%-Ch50% did not counteract DSS-induced stress.

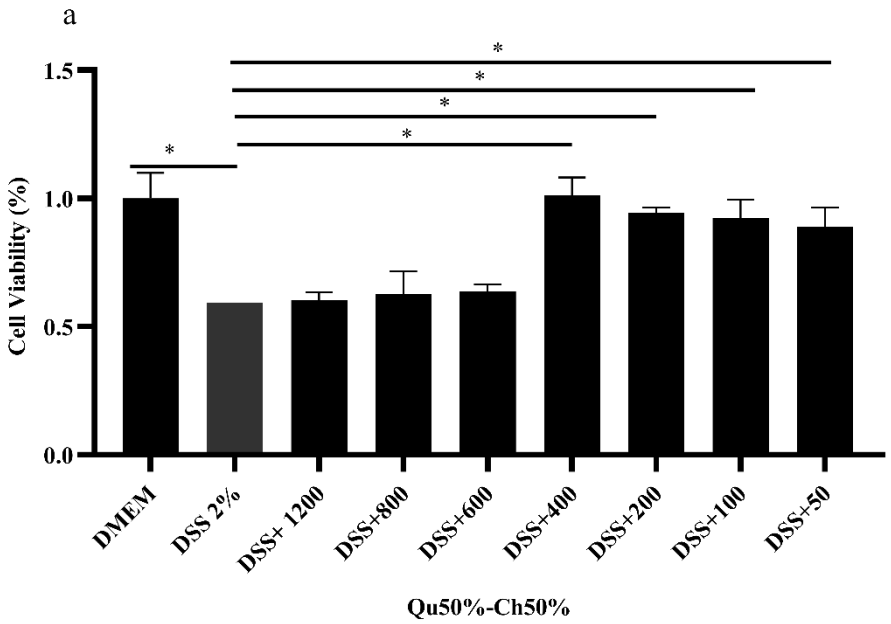

Figure 6. *Cont.*

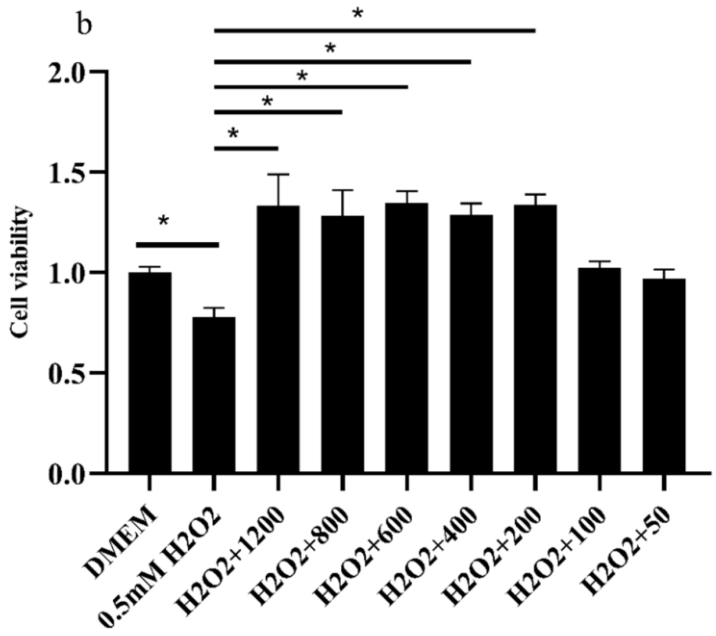

Figure 6. Effect of 3-h pre-treatment with different concentrations (50–1200 μg/mL) of *in vitro* digested Qu50%–Ch50% on IPEC-J2 cells further stressed with 2% DSS for 24 h (**a**) or 0.5 mM of hydrogen peroxide (H_2O_2) (**b**) on the metabolic activity (expressed as cell viability, MTT assay). Data are expressed as lsmeans ± SEM (n = 3, one-way ANOVA). * Denote significant differences ($p < 0.05$).

In the H_2O_2 -challenged IPEC-J2 cells (Figure 6b), the 3-h pre-treatment with Qu50%–Ch50% mitigated the oxidative stress experimentally induced by increasing cell viability. In particular, Qu50%–Ch50% at the highest concentrations tested (1200–200 μg/mL) significantly counteracted the stress induced by 0.5 mM of H_2O_2.

4. Discussion

The principal objectives of this study were to assess the AOX capacity and *E. coli* growth inhibitory activity of Qu, Ch, and their mixtures after solubilization in water and after *in vitro* digestion. We also aimed to determine whether tannin digests have a trophic effect on swine intestinal epithelial IPEC-J2 cell viability.

The AOX capacity results from our study revealed that Ch and Qu25%–Ch75% showed the highest AOX capacity compared with Qu and with all the other tannin mixtures. In the combined samples, the presence of Ch dose-dependently increased the AOX capacity and the combination of high doses of Ch (75%) with low doses of Qu (25%) showed the highest AOX effect. Comparing our data with those reported by Pèrez-Burillo et al. [29,30], our samples showed an over 30-fold higher antioxidant capacity than the conventional food and feed ingredients analysed in their study. The high reducing capacity of Ch and Qu could be attributed to the high concentration of phenolic compounds in tannin extracts. On the other hand, *in vitro* digested Ch and Qu50%–Ch50% showed a higher antioxidant activity compared to Qu. Our data are in line with those of Molino et al. [31] who reported a higher antioxidant activity for Ch (8.16 mmol Trolox/g) compared with Qu (6.70 mmol Trolox/g) after *in vitro* digestion. In general, compared to our results, they reported higher values of antioxidant capacity in their samples, which may be due to the different methodology used for antioxidant evaluation (GAR method) and the different *in vitro* digestion protocol used in their study.

Our *E. coli* growth inhibitory activity results demonstrated that Ch and Qu tannins, at specific concentrations and time, inhibit the growth of E. coli F4+ and F18+ *in vitro*. The Ch rapidly (from T1) became effective, while Qu seemed to exhibit a stable growth inhibitory activity only after three hours of incubation with E. coli. However, Qu E. coli growth inhibitory activity was maintained until the end of our analysis (6 h).

These results suggest that the rapid effect of Ch observed was associated with the more prolonged effect of Qu. Generally, under our culture conditions, the combined use of Ch and Qu had a synergistic activity in the inhibition of F4+ and F18+ growth. Overall, the combinations with the highest Ch concentration (Qu50%–Ch50% and Qu25%–Ch75%) were the most effective. However, we selected the combination Qu50%–Ch50% for further analysis as it represents a balance of Ch and Qu in which the faster activity of Ch and the more prolonged activity of Qu over time are combined.

In addition, the E. coli growth inhibitory activity of Qu and of Qu50%–Ch50% against F4+ and F18+ was maintained after *in vitro* digestion, thus highlighting the possible bio-accessibility of the antimicrobial compounds in these samples. However,

the E. coli growth inhibitory activity of Ch was not maintained after digestion, which may be due to a lower bio-accessibility or to the excessive degradation of antimicrobial and antioxidant molecules in our experimental conditions.

The *E. coli* growth inhibitory activity of Ch and Qu has been evaluated in several studies. Min et al. [19] demonstrated that chestnut and mimosa tannins have growth-inhibitory and bactericidal effects *in vitro* against *E. coli* O157:H7, and chestnut tannins showed a higher bactericidal activity. Elizondo et al. [32] found that the antibacterial and antioxidant activities of Ch added to Qu tannin were higher than pure Qu but lower than Ch tannin alone. They concluded that although Ch tannin is more potent than Qu tannin, the Qu activity may remain longer in the gastrointestinal tract because of their rich condensed tannin composition. This latter point was corroborated by our results.

In fact, tannins can inhibit the growth of some pathogenic bacterial species (e.g., *E. coli*) without affecting the physiological growth and proliferation of probiotic lactic acid bacteria, which have a positive effect at the intestinal level [33]. This selective effect may be an advantage in the use of tannins in feed. The *E. coli* growth inhibition and AOX capacity of tannins may be due to the high levels of phenols in the extracts. In our experimental conditions, these functional activities were often maintained after digestion. However, these data need to be confirmed in further *in vitro* digestion tests and after total phenolic compound analysis in the digesta.

We also tested *in vitro* digested Qu, Ch and Qu50%–Ch50% on swine IPEC-J2 cell viability to determine whether E. coli growth inhibitory concentrations also affect the viability of the intestinal cells. Qu50%–Ch50% was the most effective in stimulating cell viability when administered at low concentrations. It showed a trophic effect on IPEC-J2 cells, and was therefore, used for the cell-challenging experiments.

We found that a pre-treatment of three hours with Qu50%–Ch50% mitigated the mild oxidative and inflammatory stress experimentally induced in IPEC-J2 cells. This thus highlights the potential of tannins in preventing oxidative and inflammatory conditions at the intestinal level. However, the possibility that the lowest concentrations of tannins were effective in the DSS challenge, while only the highest concentrations were effective in counteracting oxidative challenge (H_2O_2) still needs to be investigated. We suggest that the combined treatment of IPEC-J2 cells with tannins and H_2O_2 versus tannins and DSS may show a different behaviour and synergism. A limited number of studies have investigated the ability of tannins to affect intestinal cell proliferation. Brus et al. [34] reported that low doses of gallic acid increased the proliferation of IPEC-J2 cells, thus highlighting the possible role of gallic acid in the recovery of small intestinal epithelium in swine. The same group reported the ability of several commercial products containing tannins in stimulating the proliferation of

IPEC-J2 and Caco-2 cells, although at lower doses compared to our study. They also reported [35] that a water-soluble form of Ch tannin exerted beneficial effects on the small intestinal epithelial cells of chickens by stimulating the proliferation of enterocytes and increasing the antioxidant potential, with no adverse effects on cellular metabolism. Cell models of the intestine are useful as *in vitro* tools for assessing feed and food ingredients [36–39] as they represent a simplified version of the *in vivo* intestinal environment. For the safe use of tannin-based additives in feed and food, *in vitro* animal cell models can be used as a cheap and practical alternative to animal experiments to estimate the optimal dosage for further practical use.

We believe that our results corroborate the potential beneficial use of Ch and Qu, in particular in combination, in the animal diet as antimicrobial and antioxidant agents. In addition, they have a trophic effect on intestinal epithelial cells.

5. Conclusions

Our data demonstrate the ability of quebracho and chestnut tannin to exert antioxidant activity and *E. coli* growth inhibitory activity against ETEC F4+ and F18+, together with cyto-protective activity on swine intestinal epithelial cells at specific doses. After *in vitro* digestion, chestnut and quebracho showed an antioxidant capacity and above all quebracho maintained its *E. coli* growth inhibitory activity. Our data clearly demonstrate that although chestnut and quebracho had a higher *E. coli* growth inhibitory effect when administered at a high dosage, they have a trophic effect on the intestinal cell epithelium also when used at lower dosages. Based on our findings from IPEC-J2 culture, we conclude that a balanced combination of tannins (1:1) at specific dosages may produce a protective and stimulating effect on cell proliferation rather than a cytotoxic effect.

Besides suggesting the combined use of chestnut and quebracho as a strategy to maximize the inner functional effects of such compounds, our findings also indicate that the actual dosage of tannins may be key in determining their effect on bacteria and cells. Our data provide the basis for further *in vivo* studies aimed at optimizing the use of tannins as functional feed and food ingredients.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/2/195/s1, Table S1: PCR primer's conditions, Figure S1: PCR gel electrophoresis, Figure S2: Effects of (0–1200 μg/mL) of Ch and Qu tannin extracts on E. coli F4+ and F18+ growth over time (T).

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Cowan, M.M. Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. **1999**, 12, 564–582.
2. Scalbert, A. Antimicrobial properties of tannins. Phytochemistry **1991**, 30, 3875–3883.
3. Haslam, E. Natural Polyphenols (Vegetable Tannins) as Drugs: Possible Modes of Action. J. Nat. Prod. **1996**, 59, 205–215.
4. Carrasco, J.M.D.; Redondo, E.A.; Viso, N.D.P.; Redondo, L.M.; Farber, M.D.; Miyakawa, M.E.F. Tannins and Bacitracin Differentially Modulate Gut Microbiota of Broiler Chickens. BioMed Res. Int. **2018**, 2018, 1879168.
5. Buccioni, A.; Pauselli, M.; Minieri, S.; Roscini, V.; Mannelli, F.; Rapaccini, S.; Lupi, P.; Conte, G.; Serra, A.; Cappucci, A. Chestnut or quebracho tannins in the diet of grazing ewes supplemented with soybean oil: Effects on animal performances, blood parameters and fatty acid composition of plasma and milk lipids. Small Rumin. Res. **2017**, 153, 23–30.
6. Girard, M.; Hu, D.; Pradervand, N.; Neuenschwander, S.; Bee, G. Chestnut extract but not sodium salicylatebdecrease the severity of diarrhea and enterotoxigenic Escherichia coli F4 shedding in artificially infected piglets. bioRxiv **2019**, 575662.
7. Henke, A.;Westreicher-Kristen, E.; Molkentin, J.; Dickhoefer, U.; Knappstein, K.; Hasler, M.; Susenbeth, A. Effect of dietary quebracho tannin extract on milk fatty acid composition in cows. J. Dairy Sci. **2017**, 100, 6229–6238.
8. Girard, M.; Thanner, S.; Pradervand, N.; Hu, D.; Ollagnier, C.; Bee, G. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental ETEC F4 model. PLoS ONE **2018**, 13, e0197878.
9. Tosi, G.; Massi, P.; Antongiovanni, M.; Buccioni, A.; Minieri, S.; Marenchino, L.; Mele, M. Efficacy test of a hydrolysable tannin extract against necrotic enteritis in challenged broiler chickens. Ital. J. Anim. Sci. **2013**, 12, 386–389.

10. Serrano, J.; Puupponen-Pimiä, R.; Dauer, A.; Aura, A.M.; Saura-Calixto, F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. Mol. Nutr. Food Res. **2009**, 53, 310–329.
11. Rossi, L.; Vagni, S.; Polidori, C.; Alborali, G.L.; Baldi, A.; Dell'Orto, V. Experimental Induction of Escherichia coli Diarrhoea in Weaned Piglets. Open J. Vet. Med. **2012**, 2, 1–8.
12. Rossi, L.; Di Giancamillo, A.; Reggi, S.; Domeneghini, C.; Baldi, A.; Sala, V.; Dell'Orto, V.; Coddens, A.; Cox, E.; Fogher, C. Expression of verocytotoxic Escherichia coli antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. J. Vet. Sci. **2013**, 14, 263–270.
13. Nagy, B.; Fekete, P.Z. Enterotoxigenic Escherichia coli in veterinary medicine. Int. J. Med. Microbiol. **2005**, 295, 443–454.
14. Rossi, L.; Dell'Orto, V.; Vagni, S.; Sala, V.; Reggi, S.; Baldi, A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic Escherichia coli strain in piglets. Vet. Res. Commun. **2014**, 38, 39–49.
15. Melkebeek, V.; Goddeeris, B.M. ETEC vaccination in pigs. Vet. Immunol. Immunopathol. **2013**, 152, 37–42.
16. Huang, Q.; Liu, X.; Zhao, G.; Hu, T.; Wang, Y. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. Anim. Nutr. **2018**, 4, 137–150.
17. Association of Official Analytical Chemists (AOAC). Official Methods of Analysis, 18th ed.; AOAC Int.: Gaithersburg, MD, USA, 2005.
18. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. **1999**, 26, 1231–1237.
19. Min, B.R.; Attwood, G.T.; McNabb,W.C.; Molan, A.L.; Barry, T.N. The effect of condensed tannins from Lotus corniculatus on the proteolytic activities and growth of rumen bacteria. Anim. Feed Sci. Technol. **2005**, 121, 45–58.
20. Zhang, X.;Wang, Y.; Guo, J.; Yu, Y.; Li, J.; Guo, Y.; Liu, C. Comparing two functions for optical density and cell numbers in bacterial exponential growth phase. J. Pure Appl. Microbiol. **2015**, 9, 299–305.
21. Collins, C.H.; Lyne, P.M.; Grange, J. Collins and Lyne's Microbiological Methods; Butterworth-Heinemann: Oxford, UK, 1995; Volume 493.
22. Alade, P.I.; Irobi, O.N. Antimicrobial activities of crude leaf extracts of Acalypha wilkesiana. J. Ethnopharmacol. **1993**, 39, 171–174.
23. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; et al. A standardised static *in vitro* digestion method suitable for food-an international consensus. Food Funct. **2014**, 5, 1113–1124.
24. Castrica, M.; Rebucci, R.; Giromini, C.; Tretola, M.; Cattaneo, D.; Baldi, A. Total phenolic content and antioxidant capacity of agri-food waste and by-products. Ital. J. Anim. Sci. **2019**, 18, 336–341.
25. Giromini, C.; Fekete, Á.; Givens, D.; Baldi, A.; Lovegrove, J. Short-Communication: A Comparison of the *In Vitro* Angiotensin-1-Converting Enzyme Inhibitory Capacity of Dairy and Plant Protein Supplements. Nutrients **2017**, 9, 1352.
26. Verhoeckx, K.; Cotter, P.; López-Expósito, I.; Kleiveland, C.; Lea, T.; Mackie, A.; Requena, T.; Swiatecka, D.; Wichers, H. (Eds.) The Impact of Food Bioactives on Health: *In Vitro* and ex Vivo Models; Springer: Cham, Switzerland, 2015; pp. 125–134.

27. Vergauwen, H.; Tambuyzer, B.; Jennes, K.; Degroote, J.; Wang, W.; De Smet, S.; Michiels, J.; Van Ginneken, C. Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an *In Vitro* model for the porcine gastrointestinal tract. PLoS ONE **2015**, 10, e0120485.
28. Sundaram, T.S.; Giromini, C.; Rebucci, R.; Baldi, A. Establishment of inflammatory *in vitro* intestinal epithelial models for translational animal nutrition. Ital. J. Anim. Sci. **2018**, 18, P093. 29. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an improved Global Antioxidant Response method (GAR+): Physiological-resembling *in vitro* antioxidant capacity methods. Food Chem. **2018**, 239, 1263–1272.
29. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an improved global antioxidant response
30. method (GAR+): Physiological-resembling *in vitro* digestion-fermentation method. Food Chem. **2018**, 239, 1253–1262.
31. Molino, S.; Fernández-Miyakawa, M.; Giovando, S.; Rufián-Henares, J.Á. Study of antioxidant capacity and metabolization of quebracho and chestnut tannins through *in vitro* gastrointestinal digestion-fermentation. J. Funct. Foods **2018**, 49, 188–195.
32. Elizondo, A.M.; Mercado, E.C.; Rabinovitz, B.C.; Fernandez-Miyakawa, M.E. Effect of tannins on the *in vitro* growth of Clostridium perfringens. Vet. Microbiol. **2010**, 145, 308–314. 33. Girard, M.; Bee, G. Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. Animal **2020**, 14, 95–107.
33. Brus, M.; Langerholc, T.; Škorjanc, D. Effect of hydrolysable tannins on proliferation of small intestinal porcine and human enterocytes. Acta Agric. Slov. **2013**, 4, 131–134.
34. Brus, M.; Gradišnik, L.; Trapeˇcar, M.; Škorjanc, D.; Frangež, R. Beneficial effects of water-soluble chestnut (Castanea sativa Mill.) tannin extract on chicken small intestinal epithelial cell culture. Poult. Sci. **2018**, 97, 1271–1282.
35. Giromini, C.; Cheli, F.; Rebucci, R.; Baldi, A. Invited review: Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. J. Dairy Sci. **2019**, 102, 929–942.
36. Fusi, E.; Giromini, C.; Rebucci, R.; Pinotti, L.; Caprarulo, V.; Cheli, F.; Vitari, F.; Domenighini, C.; Baldi, A. Ochratoxin A cytotoxicity on Madin—Darby canine kidney cells in the presence of alpha-tocopherol: Effects on cell viability and tight junctions. J. Anim. Physiol. Anim. Nutr. **2018**, 102, 350–355.
37. Giromini, C.; Rebucci, R.; Fusi, E.; Rossi, L.; Saccone, F.; Baldi, A. Cytotoxicity, apoptosis, DNA damage and methylation in mammary and kidney epithelial cell lines exposed to ochratoxin A. Cell Biol. Toxicol. **2016**, 32, 249–258.
38. Giromini, C.; Baldi, A.; Fusi, E.; Rebucci, R.; Purup, S. Effect of growth factors, estradiol 17-β, and short chain fatty acids on the intestinal HT29-MTX cells: Growth factors and SCFAs effects on intestinal E12 cells. Cell Biol. Toxicol. **2015**, 31, 199–209.

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Evaluation of Adhesive Characteristics of *L. plantarum* and *L. reuteri* Isolated from Weaned Piglets

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Brief Introduction to the Study:
This preliminary study was fundamental for showing encouraging results, which led to the further *in vivo* evaluation of the two probiotic strains considered in this paper in pigs. In addition, the importance of the study relies on the use of alternative methods to animal experimentation to assess the probiotic characteristics in order to predict the efficacy of bacterial strains *in vivo* without the use of animals according to the 3R principles on animal experimentation. The evaluation of *L. plantarum* and *L. reuteri* strains made possible to underline their main probiotic capacities using several *in vitro* techniques. The study demonstrated the importance of surface proteins in adhesive ability and pathogens' competition capacity of *L. plantarum* and *L. retueri* highlighting their potential dietary supplementation for promoting proper intestinal eubiosis in the host. The study was fundamental for its propaedeutic before the *in vivo* evaluation of the following microbial strains, demonstrating their potential as probiotic species for pig nutrition. In addition, the study highlighted the adhesive capacities of *L. reuteri* and *L. plantarum* without the use of animals in line with the reduction principle of animal experimentation.

3.3 Evaluation of Adhesive Characteristics of *L. plantarum* and *L. reuteri* Isolated from Weaned Piglets

Matteo Dell'Anno [1], Carlotta Giromini [1,*], Serena Reggi [1], Mariagrazia Cavalleri [1], Alessandra Moscatelli [2], Elisabetta Onelli [2], Raffaella Rebucci [1], Tamil Selvi Sundaram [1], Simona Coranelli [3], Ambra Spalletta [3], Antonella Baldi [1] and Luciana Rossi [1]

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Abstract: *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum* strains, previously isolated from weaned piglets, were considered for the evaluation of their adhesive characteristics. Lactobacilli were treated with LiCl in order to remove the surface protein layer, and probiotic activity was compared with those of untreated strains. The autoaggregation, co-aggregation to *E. coli* F18+, and adhesive abilities of LiCl-treated *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum* were significantly inhibited ($p < 0.05$) compared with the respective untreated strain. The hydrophobic and basic phenotypes were observed due to the strong affinity to chloroform and low adherence to ethyl acetate. In particular, *L. plantarum* showed higher hydrophobicity compared to *L. reuteri*, which may reflect their different colonizing ability. After treatment with LiCl to remove surface proteins, the adherence capabilities of *L. reuteri* and *L. casei* on IPEC-J2 cells decreased significantly ($p < 0.001$) and *L. reuteri* adhered more frequently. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that both *L. reuteri* and *L. plantarum* had several bands ranging from 20 to 100 kDa. Two-dimensional gel electrophoresis showed an acidic profile of the surface-layer polypeptides for both bacterial strains, and more studies are needed to characterize their profile and functions. The results confirm the pivotal role of surface proteins in the probiotic potential of *L. reuteri* and *L. plantarum*.

Keywords: probiotic; surface proteins; intestinal adhesion; gastro-intestinal system; *E. coli*; F18; pig; *Lactobacillus plantarum*; *Lactobacillus reuteri*; IPEC-J2

1. Introduction

Lactobacilli are part of the common flora in the porcine digestive tract [1]. Niu et al. [2] showed that the *Lactobacillus* genus accounts for approximately 15% of 16S rRNA in intestinal pig samples, regardless of age. Notably, while the swine faecal microbiota changed significantly across growth stages, the populations of lactobacilli remain almost stable.

Although several studies have shown the positive impact of *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum*, previously known as the *Lactobacillus* genus, on piglet's performance improvement, diarrhoea prevention, stress alleviation, immunity, microbiota modulation, and carcass quality [3,4], they are not listed in the European register of additives for pigs. Moreover, the species- and strain-specific characteristics of lactobacilli that confer probiotic benefits are still not well-documented. Indeed, *L. plantarum* can be found in the European Register of Feed Additives according to European Regulation [5], under the categories of preservatives, silage additives, microorganisms, and gut flora stabilizers for fattening chickens. On the other hand, *L. reuteri* is mostly used as a probiotic in humans. Lactobacilli are

generally selected as potential probiotics due to their natural ability to survive the digestive process. After the arrival in the intestinal section, lactobacilli can protect the host from pathogens by means of different mechanisms (competitive exclusion, bacteriocins production and stimulation of mucosal immunity) [6,7]. The adhesive capacity of bacteria to epithelial cells is one of the main probiotic characteristics [8]. The lactobacilli are Gram-positive bacteria characterized by a cell envelope consisting of a cell membrane and cell wall. These two layers are covered by several surface proteins, with the main function being as a protective sheath against environmental challenges. It has been previously proposed that bacteria surface proteins of lactobacilli are involved in cell protection and surface recognition and that they could be potential mediators of bacteria autoaggregation and adhesion to intestinal cells [9]. *L. reuteri* and *L. plantarum* were previously demonstrated to have a significant role in controlling diarrhoea in piglets [10]. In particular, dietary supplementation of 2×10^8 CFU/g of *L. plantarum* and *L. reuteri* significantly reduced diarrhoea occurrence and had the lowest faecal score in our trial. *L. plantarum* showed the lowest diarrhoea frequency compared to the other bacterial strains and their combinations. *L. plantarum* and *L. reuteri* supplementation did not influence animal performance, total faecal bacteria, faecal lactobacilli and coliform. In addition, *L. plantarum* and *L. reuteri* showed high resistance to a wide range of pH and digestive processes [10]. In our attempt to study the adhesive characteristics in the probiotic activity of *L. reuteri* and *L. plantarum*, we investigated *Limosilactobacillus reuteri* and *Lactiplantibacillus plantarum* autoaggregation, co-aggregation capacity to *Escherichia coli* F18+, bacterial hydrophobicity, adhesion to swine intestinal IPEC-J2 cells and cell surface proteins characteristics.

2. Materials and Methods

2.1. Bacterial Strains and Culturing Conditions

Single colonies of *L. plantarum* and *L. reuteri*, isolated from swine, were obtained from the Biotecnologie B.T. (Perugia, Italy) strain collection [10] and grown in MRS agar medium. *L. plantarum* and *L. reuteri* strains were individually inoculated from our laboratory stock at −80 °C into the De Man, Rogosa and Sharpe (MRS) medium (Liofilchem, Italy) and incubated at 35 °C for 24 h under a microaerophilic atmosphere by adding sterile oil above the culture media.

2.2. Aggregative Abilities of LAB Strains

Lactobacilli were cultured in MRS broth at 35 °C for 24 h under a microaerophilic atmosphere by adding sterile oil above the culture media. Bacterial pellets were harvested by centrifugation (3500 rpm, 10 min), and cells were rinsed with PBS (1X) in order to reach an optical density (OD) of 0.2–0.3 at 600 nm wavelength using a spectrophotometer (Jasco V630 UV-VIS, JASCO Deutschland GmbH, Pfungstadt, Germany). Cell suspensions were vortexed for 6 s and incubated 6 h at room

temperature. On millilitre of the upper part of the suspension was measured at 600 nm each hour. The percentage of auto-aggregation was then calculated.

$$\%\ autoaggregation = \left[1 - \left(\frac{\mathrm{At}}{\mathrm{A0}}\right)\right] \times 100$$

where At is the absorbance at different time points and A0 the initial one.

For co-aggregation abilities, resuspended LAB strains were mixed with aliquots of F18+ *E. coli* previously characterized for the presence of F18 adhesive fimbriae [11]. The *E. coli* culture was obtained by incubating the bacterial strain at 37 °C in Luria–Bertani medium under aerophilic conditions, and to left stir overnight at 110 rpm. Samples were mixed thoroughly for 10 s and incubated for 6 h at room temperature. One millilitre of the upper part of the suspension was measured at 600 nm each hour. The co-aggregation percentage was then calculated.

$$\%\ \mathrm{coaggregation} = \left\{ \frac{\left[\left(\frac{\mathrm{Ax} + \mathrm{Ay}}{2}\right) - \mathrm{A\ (x + y)}\right]}{\mathrm{Ax} + \mathrm{Ay}/2} \right\} \times 100$$

Ax and Ay indicate the individual proprieties of lactobacilli and *E. coli*, and A(x+y) express the combined aggregation of *L. plantarum* or *L. reuteri* and *E. coli*. The whole analyses were performed in two independent experiments including three replicates.

2.3. Determination of Bacterial Hydrophobicity

Microbial adhesion to solvents (MATS) was assessed according to Kos et al. [12]. Briefly, bacteria were harvested from the stationary phase after centrifugation (5000× *g* for 15 min). Samples were washed twice and resuspended in PBS (1X, pH 7.0) and brought to OD 0.6 at 600 nm. For the assay, 1 mL of the solvent was added to 3 mL of the bacterial suspension. After 10 min as preincubation at room temperature, samples were mixed for 2 min. After 20 min, the aqueous phase was removed to measure the OD at 600 nm (A1). The percentage of bacterial surface hydrophobicity was then calculated.

$$\left(\frac{\text{Absorbance before mixing} - \text{Absorbance after mixing}}{\text{Absorbance before mixing}}\right) \times 100$$

For the evaluation of surface hydrophobicity, three solvents were tested: toluene (Titolchimica, Italy) as the apolar solvent; chloroform (Merck, Darmastadt, Germany) as monopolar basic and acidic solvent; and ethyl acetate (Carlo Erba Reagents S.A.S, Milan, Italy) as monopolar basic solvent [13]. The whole analyses were performed in two independent experiments including three replicates.

2.4. Cell Line and Culture Conditions

IPEC-J2 is a non-transformed cell line, derived from the jejunum epithelium of unsuckled piglets (DSMZ, Braunschweig, Germany). Cells used for the experiment were defrosted from a cryopreserved stock and cell passages of 24–28 were used for the experiments. Cells were routinely grown in a total volume of 100 mL of 1:1 of Dulbecco's modified Eagle's medium with stable L-glutamate and Ham's F-12 mixture (DMEM/F12) (Immunological sciences, Società Italiana Chimici, Rome, Italy), plus 15 mM of HEPES (Sigma-Aldrich, Milano, Italy), 5% heat-inactivated foetal bovine serum (FBS) (Immunological sciences, Società Italiana Chimici, Rome, Italy), 1% penicillin (100 U/mL)/streptomycin (100 mg/mL) (Euroclone, Milano, Italy) and 1% GlutaMAX at 37 °C in a 5% CO_2 atmosphere and subcultivated at 80% confluence. For adhesion assay, IPEC-J2 monolayers were prepared in a 2-well chamber slides system coated with collagen. After collagen coating, the cells were seeded at a concentration of 4 × 10^5 cells/chamber to reach 80% confluence before lactobacilli addition.

2.5. Bacterial Adhesion Assay

Two-well chamber slides were used to study the adhesion ability of *L. plantarum*, *L. reuteri* and *L. casei* ATCC 393 (reference strain) to IPEC-J2 cells. Two millilitres of 48 h cultures of *L. plantarum*, *L. reuteri* and *L. casei* grown in 30 mL MRS broth (adjusted to 2.3 × 10^8 CFU/mL) were centrifuged, washed, and resuspended in DMEM/F12 medium. Before the experiments, the cell medium was removed and 1 mL of each bacterial suspension was added to one well of each chamber slide, while the other well was filled with 1 mL of each bacterial suspension after LiCl (5 M) treatment [14]. Briefly, the cell pellet was resuspended in LiCl (5 M) and incubated at room temperature for 30 min. Bacteria were centrifugated at 5000× *g* for 15 min, and the cell pellet was washed twice with sterile saline solution before being resuspended in the cell medium. After 1 h incubation in a 5% CO_2 atmosphere, the chamber slides were washed twice with PBS (1X) with Ca and Mg, and a 15 min fixation step with 500 µL of MetOH was made. Then, GIEMSA staining was performed with the GIEMSA dye diluted at a ratio of 1:20 with PBS (1X) for 30 min. After washing with deionized water, the glasses were left to dry overnight. Cells and bacteria in a 20 microscopy field (100× magnification with oil immersion) were randomly counted**;** the bacteria adherent to at least 200 cells were counted. The analyses were performed in three independent experiments including at least two technical replicates per treatment. The bacterial adherence value was defined as the number of the adhered bacteria per cell [15]. Data are expressed as adherent bacteria/number of cells.

2.6. Isolation and One and Two-Dimensional Gel Electrophoresis of S-Layer Proteins from L. plantarum and L. reuteri

S-layer proteins of lactobacilli were extracted by 5 M LiCl according to the method reported by Singh et al. [16]. Briefly, lactobacilli were incubated in 30 mL MRS at 35 °C in anaerobiosis conditions. Cells were collected and washed twice with sterile water. The pellet was treated with 5 M LiCl at 4 °C for 30 min. The supernatant was collected and dialyzed with PBS (1X) and concentrated. The extracted surface proteins were quantified using the Bradford method, with bovine serum albumin (BSA) as the standard [17]. For determination of the apparent molecular mass, SDS-PAGE was performed using a 10% (*w*/*v*) acrylamide gel in denaturing and reducing conditions. The gel was stained by Coomassie brilliant blue R-250 (Sigma, Saint Louis, Missouri, USA) and the intensity of bands was evaluated using ImageJ software. For two-dimensional gel electrophoresis, 25 μg of extracted S-layer proteins were precipitated with cold ethanol (1:5 *v*/*v*) and incubated for 30 min in ice. The pellet was obtained by centrifugation at 4 °C for 36 min at 20,627 *g* (15,000 rpm) in an ALC A21-C rotor. Ethanol was removed and the pellet was resuspended with 2D 200 μL of rehydration buffer (8 M urea, 4% CHAPS, 65 mM dithioerythritol, 0.5% bromophenol blue) supplemented with 2% 3–10 IPG buffer and loaded onto 7-cm non-linear pH 3–10 strips by overnight passive rehydration at room temperature. IEF was performed with Multifor II system (GE Healthcare, Chicago, IL, USA) according to Moscatelli et al. [18] (200 V for 1 h, 2000 V for 3 h and 3000 V for 3 h and 30 min). Focused strips were equilibrated in Buffer I (0.5 M TrisHCl, pH 6.8, 2% SDS, 6 M urea, 30% glycerol, 2% DTE) for 12 min and then for another 5 min in Buffer II (composition the same as Buffer I, but with 2.5% iodoacetamide instead of DTE) at room temperature. SDS-PAGE was run in 10% polyacrylamide gel (MiniVe Vertical Electrophoresis System, GE Healthcare, USA), as described previously [19]. The gels were silver-stained [20].

2.7. Statistical Analysis

The statistical analyses were performed through the software GraphPad Prism 9.0.1. The results of the aggregation, co-aggregation, cell surface hydrophobicity and adhesion assay for both the species were analysed through a one-way analysis of variance (ANOVA) procedure. Pairwise comparisons were evaluated using Tukey's HSD test. Differences were considered statistically significant at $p \leq 0.05$. Data were expressed as the least squares (LS) means ± standard error of mean (SEM).

3. Results

3.1. Aggregation Abilities

The auto-and co-aggregation abilities of the *L. plantarum* and *L. reuteri* are summarized in Figures 1 and 2. After 6 h of incubation, the highest percentages of aggregation were observed for *L. reuteri*. Both strains demonstrated co-aggregation ability with intestinal pathogen tested (*E. coli* F18+). The maximum autoaggregation and co-aggregation were shown by *L reuteri* at 6 h (38.46 ± 0.49% and 11.60 ± 0.30%, respectively). The auto-aggregation and co-aggregation ability to *E. coli* F18+ of *L. reuteri* decreased significantly after LiCl treatment compared to the untreated strain ($p < 0.05$), while *L. plantarum* showed that LiCl treatment did not impair its co-aggregation ability to *E. coli* (Figure 3).

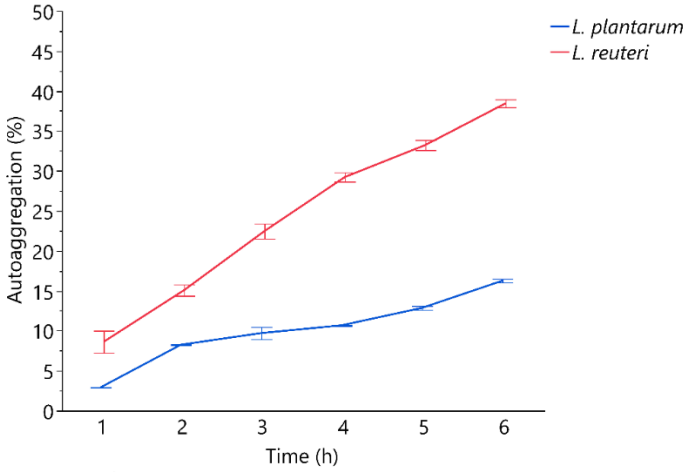

Figure 1. Percentages of autoaggregation measured at hourly bases. Data are presented as means ± standard deviations.

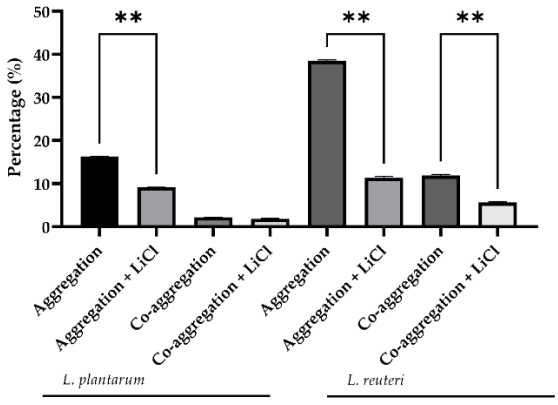

Figure 2. Percentage of *L. reuteri* and *L. plantarum* aggregation and co-aggregation with *E. coli* F18 measured at 6 h. Data are presented as LS mean ± SEM. ** indicates a statistically significant difference ($p \leq 0.001$).

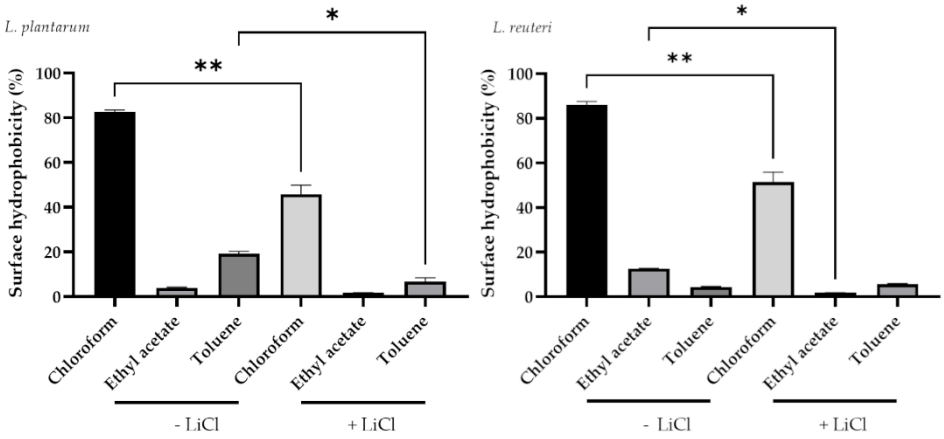

Figure 3. Effect of LiCl treatment on cell surface hydrophobicity of lactobacilli strains. Data are presented as LS means ± SEM. * Indicates a statistically significant difference ($p \leq 0.05$); ** indicates a statistically significant difference ($p \leq 0.001$).

3.2. Determination of Bacterial Hydrophobicity

In particular, *L. plantarum* and *L. reuteri* showed a significant decrease in bacteria in the aqueous phase after a chloroform treatment (82.83 ± 1.29% for untreated and 49.65 ± 1.45% for chloroform treated *L. plantarum*; 86.17 ± 2.12 for untreated and 55.22 ± 1.25% for chloroform treated *L. reuteri*; $p < 0.0001$) (Figure 3). The same drop was registered in the aqueous phase with toluene treatment for both bacterial strains (19.20 ± 1.69% for untreated and 6.91 ± 1.76% for toluene treated *L. plantarum*; 5.47 ± 1.28% for untreated and 1.94 ± 0.21% for toluene treated *L. reuteri*; $p < 0.05$).

3.3. Bacterial Adhesion Assay

L. reuteri showed the greatest adhesion capacity compared with *L. casei* and *L. plantarum* (Figures 4 and 5). *L. plantarum* adhesion was not affected by the treatment with LiCl (14.64 ± 1.22 versus 11.04 ± 1.17). On the contrary, the treatment with LiCl significantly affected the adhesion capacity of *L. reuteri* and *L. casei* ($p < 0.05$). In particular, 16.77 ± 1.82 adherent bacteria/cell were counted in *L. reuteri*-treated cells while 8.63 ± 1.08 adherent bacteria/cell in *L. reuteri* + LiCl-treated cells. Regarding *L. casei*, 10.07 ± 1.02 adherent bacteria were counted in *L. casei*-treated cells whereas 5.14 ± 0.74 adherent bacteria in *L. casei* + LiCl-treated cells.

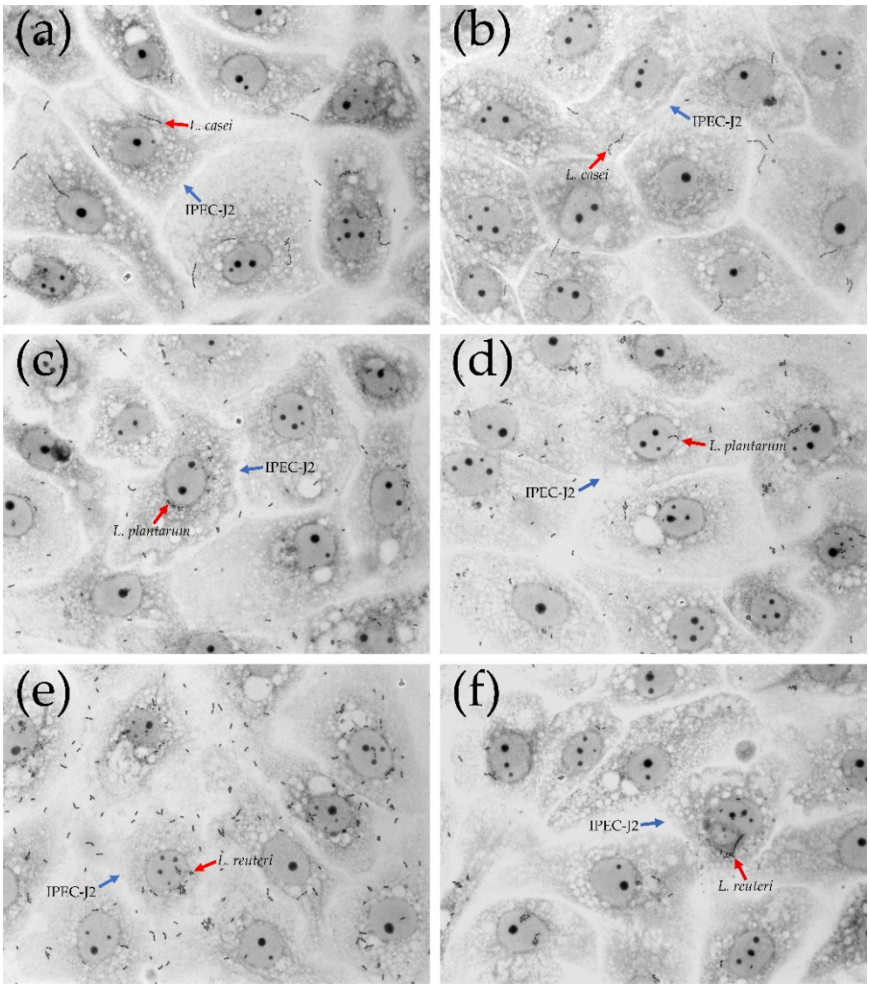

Figure 4. Adhesion of *L. casei*, *L. plantarum* and *L. reuteri* to IPEC-J2 cells. Representative microphotographs with 100× magnification (oil immersion). (**a**) *L. casei*; (**b**) *L. casei* + LiCl; (**c**) *L. plantarum*; (**d**) *L. plantarum* + LiCl; (**e**) *L. reuteri*; (**f**) *L. reuteri* + LiCl. Red arrows indicate lactobacilli strains; blue arrows indicate IPEC-J2 cells.

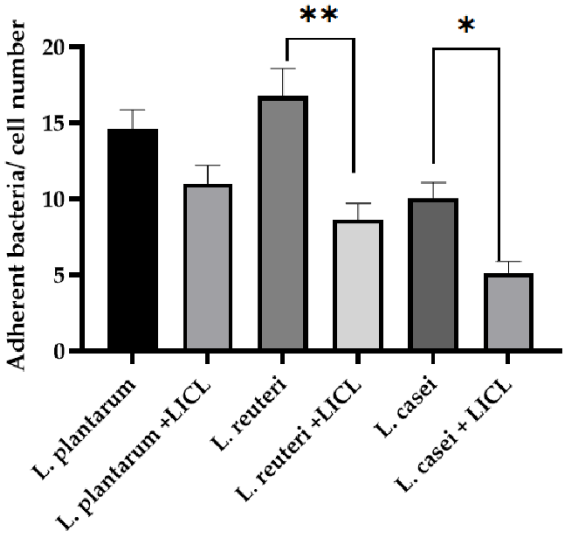

Figure 5. Count of adherent bacteria to IPEC-J2 cells, data are expressed as the mean value of adherent bacteria/cell number ± SEM of three independent experiments. * Indicates a statistically significant difference ($p \leq 0.05$); ** indicates a statistically significant difference ($p \leq 0.001$).

3.4. One and Two-Dimensional Gel Electrophoresis of Surface Proteins from Lactobacilli Strains

The extracted surface proteins resulted 1.478 μg/mL for *L. plantarum* and 0.970 μg/mL for *L. reuteri*, respectively.

One-dimensional denaturing gel revealed distinct protein bands, although quite qualitative differences in the intensity of bands of the surface proteins were observed (Figure S1). The observed polypeptides profile resulted in the range 20–100 kDa by comparison with the marker (Figure 6A). The two-dimensional gel electrophoresis did not show significant variations of protein spots between the two bacterial strains. The two-dimensional gel was mainly characterized by acid polypeptides ranging from 100 to 10 kDa for *L. plantarum* and *L. reuteri*, and no clusters were detected (Figure 6B).

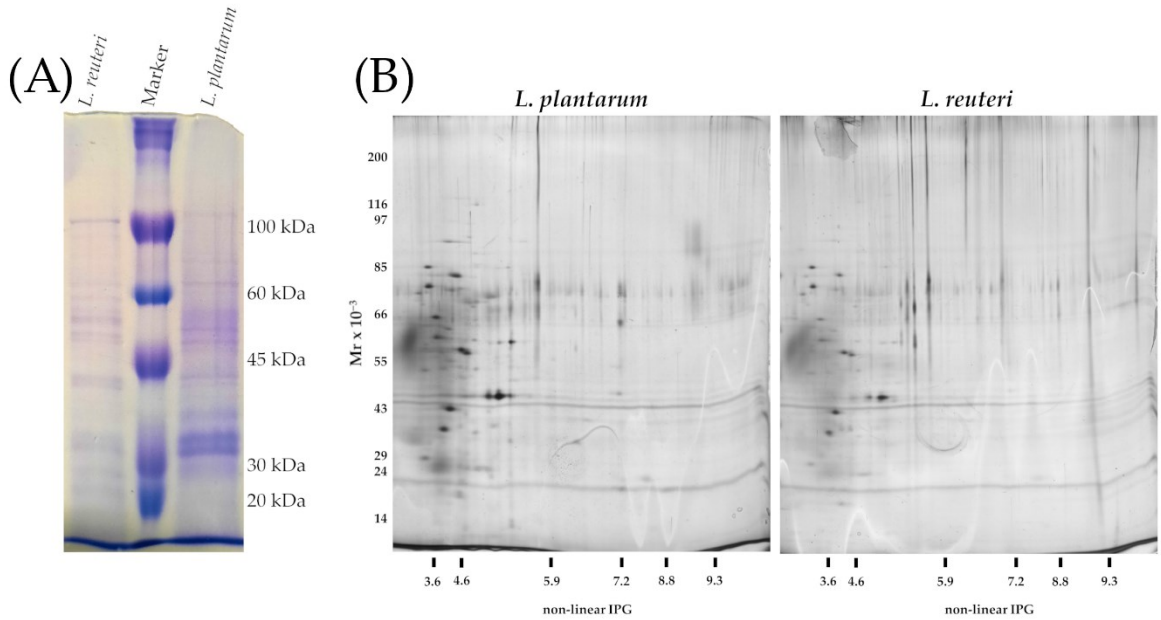

Figure 6. (**A**) SDS-PAGE of surface proteins. Equal amount of volume (14 μL) of the extracted polypeptides were loaded to each well; from left to right: S-layer extracted from *Lactobacillus reuteri*; Molecular weight marker (kDa), S-layer extracted from *Lactobacillus plantarum.* (**B**) Two-dimensional gel electrophoresis of S-layer polypeptides extracted from *L. plantarum* and *L. reuteri*.

4. Discussion

Alternatives to antibiotics are urgently needed due to the global concern regarding antibiotic resistance in livestock production systems [21–23].

Probiotics are defined as living microorganisms that can act in the treatment and prevention of infectious diseases when ingested in adequate amounts [24]. In particular, probiotic bacteria surface proteins have been demonstrated to intervene in cell protection and competitive adhesion against pathogens [9]. The presence of glyco-proteinaceous material at the bacterial cell surface is responsible of a higher

hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides and affect the binding capabilities of probiotics [9]. In our study, a hydrophobic and basic phenotype of *L. plantarum* and *L. reuteri* was observed due to lactobacilli affinity to chloroform and ethyl acetate. In particular, *L. plantarum* showed higher hydrophobicity compared to *L. reuteri*, which may reflect their different colonizing ability. The values of MATS for toluene reflect the cell surface hydrophobicity or hydrophilicity capacities. Chloroform and ethyl acetate were used to assess their characteristics as electron donors (basic) and electron acceptors (acidic) of bacteria. In this study, the use of LiCl was adopted on probiotic cells to allow the selective and efficient removal of S-layer proteins. In particular, this step was carried out to evaluate the adhesive properties without surface proteins. Firstly, hydrophobic cell surfaces are reported for autoaggregation capacities that in some cases determine the intestinal colonization [25]. As expected, the treatment with LiCl strongly affected the hydrophobicity. The observed results suggest that *L. plantarum* and *L. reuteri* could potentially adhere to gut epithelium.

Other important characteristics of a probiotic bacteria are the auto- and co-aggregation capacity. The auto-aggregation is defined as the ability of cells of the same kind to self-adhere, and is recognized as an important predictive parameter for gut epithelium colonization as it allows bacteria to maintain a significant number of cells in the environmental niche [26]. Co-aggregation is defined as the binding of bacteria of diverse species (e.g., lactobacilli versus pathogens), and also evaluates the competitive inhibition capacity [26]. In fact, the formation of co-aggregates of lactobacilli with pathogens reduced pathogenic microorganisms' ability to adhere to the intestinal epithelium [27]. In the present study, the auto-aggregation and co-aggregation to *E. coli* F18+ capacity of *L. plantarum* and *reuteri* indicate that cell surface proteins could be associated with lactobacilli aggregation properties and to specific binding capabilities in the gastrointestinal tract. Moreover, co-aggregation has been recognized as an important factor in the establishment and maintenance of a non-infectious gastrointestinal microflora. The observed co-aggregation with *E. coli* of *L. reuteri* and *L. plantarum* could suggest that lactobacilli constitute an important host-defence mechanism against infection. This hypothesis was also confirmed by the *in vivo* results described by Dell'Anno et al. [10], that registered a significative reduction in diarrhoea occurrence in weaned piglets' diets supplemented with the investigated *L. plantarum* and *L. reuteri* strains. In this study, we focused on *Escherichia coli* F18+, a widely spread porcine enterotoxigenic pathogen, responsible for important economic losses in the pig industry [28,29]. *E. coli* F18+ strain has the ability to adhere to the intestinal surface by F18 adhesive fimbriae, and it is considered a major pathogen involved in the post-weaning disease and oedema disease [30]. Our study suggests

that *L. plantarum* and *L. reuteri* can compete for adhesion with a pathogenic strain of *E. coli* through the formation of a barrier via auto-aggregation of by directly by co-aggregation with *E. coli*. It is recognized that auto-aggregation ability is related to adhesive properties of bacterial strains [31].

IPEC-J2 cell model is commonly used to investigate lactobacilli strains adhesive capacities [32]. The porcine intestinal epithelial IPEC-J2 cell line provides an excellent *in vitro* model for probiotic adhesion studies [33]. IPEC-J2 have been largely studied to assess probiotic adhesive properties and the anti-inflammatory activity of probiotic strains [34]. As shown by Tallon et al. [35], different chemical pre-treatments may influence the adhesive capacities of bacteria depending on the surface proteins that are involved in the adhesion process. In line with the study of Singh et al. [9,16], the pre-treatment with LiCl to remove cell surface proteins reduced the ability of *L. reuteri* to adhere to intestinal epithelium. On the contrary, *L. plantarum* was more resistant to LiCl treatment, probably due to the structural variations existing between the microorganism-associated molecular patterns which interact with the host pattern recognition receptors [36]. In this study, *L. reuteri* showed a higher adhesion ability compared to the behaviour of *L. casei* used as a control strain, in accordance with the study by Lähteinen et al. [37], which observed that *L. reuteri* isolates from the porcine intestine revealed the greatest adhesion capacity compared to other commensal lactobacilli in swine GIT (*L. amylovorus*, *L. mucosae* and *L. johnsonii*). In addition, specific strains of *L. reuteri* express different surface proteins capable of improving binding properties compared with a well-known probiotic such as *L. casei* [31,38–40]. Mucus binding abilities related to the production of mannose-sensitive adhesins have been reported for some *L. plantarum* and *L. reuteri* strains [25]. The observed adhesion properties could be due to the presence of mucus-binding proteins on cell surface of these two bacteria.

In line with other studies, the SDS-PAGE of cell surface proteins of *L. reuteri* and *L. plantarum* revealed the presence of several bands of varying length if compared with the protein fragments of the marker in the range from 100 to 20 kDa [9,41]. Two-dimensional gel electrophoresis confirmed the acidic prevalence of bacterial surface proteins that are typical subunits of surface proteins of lactobacilli [42]. In visual evaluation, the polypeptide profile was very similar, and the molecular range confirmed the previously observed results of one-dimensional electrophoresis (SDS-PAGE). In line with our results, Wang et al. [41] observed that a protein of approximately 37 kDa for *L. plantarum* strain. This protein, the glyceraldehyde-3phospated dehydrogenase (GAPDH), plays an important role for the adhesion properties of *L. plantarum* [41]. It has been shown that CscA, CscB and CscD proteins of *L. plantarum* are functionally related to a cell-surface protein complex that could

play a role in sugar acquisition. In particular, a cscB gene product known as co-aggregation-promoting factor (Cpf) could be removed from *L. plantarum* surface by treating bacterial cells with LiCl (5M) and reattached by salt removal, restoring its co-aggregation ability [43]. Mannose-binding proteins of *L. plantarum* WCFS1 are similar to a mucus-binding protein from *L. reuteri* that are likely to be involved in the interaction with the host [44]. *L. reuteri* 100-23 possesses a high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB), which both contribute to adherence in the gut [45]. *L. reuteri* JCM1081's adhesive property appeared to be mediated by the presence of a surface protein of approximately 29 kDa with an important similarity to the putative ATP-binding cassette transported protein CnBP [46]. In our study, *L. plantarum* and *L. reuteri* revealed a quite similar surface profile, with polypeptides ranging from 20 to 100 kDa; however, their characterization and functions need to be confirmed by further experiments.

The survival of probiotics during gastrointestinal transit and their adhesion on the intestinal surface are important prerequisites for the colonization and competitive inhibition proprieties. The strains here analysed have been previously demonstrated to greatly survive the gastro-intestinal conditions [10], which is an important prerequisite in probiotic efficacy. The results here presented clearly indicate the relation between cell surface characteristics and adhesion ability of *L. reuteri* and *L. plantarum.* Therefore, more studies are needed to identify the particular proteins involved in these mechanisms, in order to select the most suitable probiotic strains for application in weaning piglets. The findings of the current study can be extrapolated to those situations in which it could be relevant to formulate a probiotic product to target performance or health-related challenges in the pig's life.

5. Conclusions

Our study showed that LiCl significantly inhibited the autoaggregation and co-aggregation to *E. coli* of *L. plantarum* and *L. reuteri*. In addition, a strong affinity to chloroform indicated the hydrophobic and basic phenotypes of these lactobacilli. The adhesive capacity of *L. reuteri* on IPEC-J2 cells was significantly reduced after a pre-treatment with LiCl, suggesting a pivotal role of surface proteins in the epithelial adhesion. *L. plantarum* and *L. reuteri* revealed an important influence of the adhesive proteins related to their probiotic characteristics, further research is necessary to better address the mechanisms of these proteins in the gut colonization, and also in relation to intestinal microbiota. Additionally, *L. plantarum* and *L. reuteri* might be suitable candidates for further study, due to their protective effects against *E. coli* infections in weaned piglets.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article.

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References

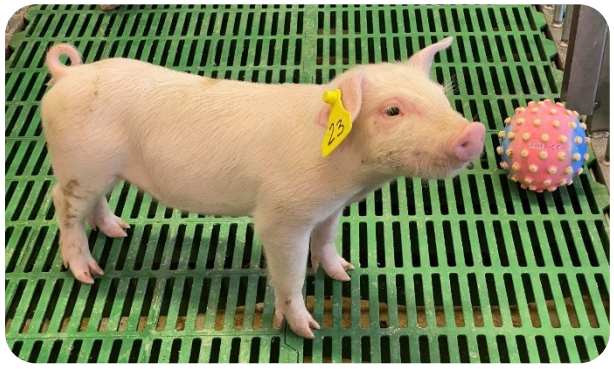
1. Valeriano, V.D.; Balolong, M.; Kang, D.-K.; Valeriano, V.D.; Balolong, M.; Kang, D.-K. Probiotic roles ofLactobacillussp. in swine: Insights from gut microbiota. *J. Appl. Microbiol.* **2017**, *122*, 554–567, doi:10.1111/jam.13364.
2. Niu, Q.; Li, P.; Hao, S.; Zhang, Y.; Kim, S.W.; Li, H.; Ma, X.; Gao, S.; He, L.; Wu, W. Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Sci. Rep.* **2015**, *5*, 1–7.
3. Hou, C.; Zeng, X.; Yang, F.; Liu, H.; Qiao, S. Study and use of the probiotic Lactobacillus reuteri in pigs: A review. *J. Anim. Sci. Biotechnol.* **2015**, *6*, 1–8, doi:10.1186/s40104-015-0014-3.
4. Wang, W.; Chen, J.; Zhou, H.; Wang, L.; Ding, S.; Wang, Y.; Song, D.; Li, A. Effects of microencapsulated *Lactobacillus plantarum* and fructooligosaccharide on growth performance, blood immune parameters, and intestinal morphology in weaned piglets. *Food Agric. Immunol.* **2017**, *29*, 84–94, doi:10.1080/09540105.2017.1360254.

5. Regulation EC 1831/2003. of the European Parliament and of the Council, of 22 September 2003 on Additives for Use in Animal Nutrition (Text with EEA Relevance), Official Journal of the European Union, Brussels, Belgium.
6. Walter, J. Ecological Role of Lactobacilli in the Gastrointestinal Tract: Implications for Fundamental and Biomedical Research. *Appl. Environ. Microbiol.* **2008**, *74*, 4985–4996, doi:10.1128/aem.00753-08.
7. Hekmat, S.; Soltani, H.; Reid, G. Growth and survival of Lactobacillus reuteri RC-14 and Lactobacillus rhamnosus GR-1 in yogurt for use as a functional food. *Innov. Food Sci. Emerg. Technol.* **2009**, *10*, 293–296, doi:10.1016/j.ifset.2008.10.007.
8. Van Tassell, M.L.; Miller, M.J. Lactobacillus Adhesion to Mucus. *Nutrients* **2011**, *3*, 613–636, doi:10.3390/nu3050613.
9. Singh, T.P.; Malik, R.K.; Kaur, G. Cell surface proteins play an important role in probiotic activities of Lactobacillus reuteri. *Nutrire* **2016**, *41*, 1–10.
10. Dell'Anno, M.; Callegari, M.L.; Reggi, S.; Caprarulo, V.; Giromini, C.; Spalletta, A.; Coranelli, S.; Rossi, C.A.S.; Rossi, L. Lac-tobacillus plantarum and Lactobacillus reuteri as Functional Feed Additives to Prevent Diarrhoea in Weaned Piglets. *Animals* **2021**, *11*, 1766.
11. Dell'Anno, M.; Sotira, S.; Rebucci, R.; Reggi, S.; Castiglioni, B.; Rossi, L. *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts. *Ital. J. Anim. Sci.* **2020**, *19*, 103–113, doi:10.1080/1828051x.2019.1703563.
12. Kos, B.; Šušković, J.; Vuković, S.; Šimpraga, M.; Frece, J.; Matošić, S. Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. *J. Appl. Microbiol*. **2003**, *94*, 981–987.
13. Bellon-Fontaine, M.-N.; Rault, J.; Van Oss, C. Microbial adhesion to solvents: A novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids Surfaces B Biointerfaces* **1996**, *7*, 47–53.
14. Zhang, W.; Wang, H.; Liu, J.; Zhao, Y.; Gao, K.; Zhang, J. Adhesive ability means inhibition activities for lactobacillus against pathogens and S-layer protein plays an important role in adhesion. *Anaerobe* **2013**, *22*, 97–103, doi:10.1016/j.anaerobe.2013.06.005.
15. Guan, C.; Chen, X.; Jiang, X.; Zhao, R.; Yuan, Y.; Chen, D.; Zhang, C.; Lu, M.; Lu, Z.; Gu, R. *In vitro* studies of adhesion prop-erties of six lactic acid bacteria isolated from the longevous population of China. *RSC Adv.* **2020**, *10*, 24234–24240.
16. Singh, T.P.; Kaur, G.; Kapila, S.; Malik, R.K. Antagonistic activity of Lactobacillus reuteri strains on the adhesion characteristics of selected pathogens. *Front. Microbiol*. **2017**, *8*, 486.
17. Bonjoch, N.P.; Tamayo, P.R. Protein Content Quantification by Bradford Method. In *Handbook of Plant Ecophysiology Techniques*; Springer: Berlin/Heidelberg, Germany, 2006; pp. 283–295.
18. Moscatelli, A.; Gagliardi, A.; Maneta-Peyret, L.; Bini, L.; Stroppa, N.; Onelli, E.; Landi, C.; Scali, M.; Idilli, A.I.; Moreau, P. Characterisation of detergent-insoluble membranes in pollen tubes of *Nicotiana tabacum* (L.). *Biol. Open* **2015**, *4*, 378–399, doi:10.1242/bio.201410249.
19. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685, doi:10.1038/227680a0.
20. Sinha, P.; Poland, J.; Schnölzer, M.; Rabilloud, T. A new silver staining apparatus and procedure for matrix-assisted laser desorption/ionization-time of flight analysis of proteins after two-dimensional electrophoresis. *Proteomics Int. Ed*. **2001**, *1*, 835–840.
21. Sotira, S.; Dell'Anno, M.; Caprarulo, V.; Hejna, M.; Pirrone, F.; Callegari, M.L.; Tucci, T.V.; Rossi, L. Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood

Metabolites and Gut Microbiota in Weaned Piglets. *Animals* **2020**, *10*, 726, doi:10.3390/ani10040726.
22. Zaninelli, M.; Rossi, L.; Costa, A.; Tangorra, F.; Agazzi, A.; Savoini, G. Monitoring of goats' health status by on-line analysis of milk electrical conductivity. *Large Anim. Rev*. **2015**, *21*, 81–86.
23. Dell'Anno, M.; Hejna, M.; Sotira, S.; Caprarulo, V.; Reggi, S.; Pilu, R.; Miragoli, F.; Callegari, M.L.; Panseri, S.; Rossi, L. Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets. *Anim. Feed Sci. Technol.* **2020**, *266*, 114519, doi:10.1016/j.anifeedsci.2020.114519.
24. FAO.; WHO. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. *Prevention* **2001**, *5*, 1–10.
25. Muñoz-Provencio, D.; Llopis, M.; Antolín, M.; De Torres, I.; Guarner, F.; Pérez-Martínez, G.; Monedero, V. Adhesion proper-ties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix. *Arch. Microbiol.* **2009**, *191*, 153–161.
26. Śliżewska, K.; Chlebicz-Wójcik, A.; Nowak, A. Probiotic Properties of New *Lactobacillus strains* Intended to Be Used as Feed Additives for Monogastric Animals. In *Probiotics and Antimicrobial Proteins*; Springer: Berlin/Heidelberg, Germany, 2020; pp. 1–17.
27. Krausova, G.; Hyrslova, I.; Hynstova, I. *In Vitro* Evaluation of Adhesion Capacity, Hydrophobicity, and Auto-Aggregation of Newly Isolated Potential Probiotic Strains. *Fermentation* **2019**, *5*, 100, doi:10.3390/fermentation5040100.
28. Rossi, L.; Vagni, S.; Polidori, C.; Alborali, G.L.; Baldi, A.; Dell'Orto, V. Experimental Induction of *Escherichia coli* Diarrhoea in Weaned Piglets. *Open J. Veter-Med.* **2012**, *2*, 1–8, doi:10.4236/ojvm.2012.21001.
29. Rossi, L.; Pinotti, L.; Agazzi, A.; Dell'Orto, V.; Baldi, A. Plant bioreactors for the antigenic hook-associated flgK protein expression. *Ital. J. Anim. Sci.* **2014**, *13*, 2939.
30. Rossi, L.; Dell'Orto, V.; Vagni, S.; Sala, V.; Reggi, S.; Baldi, A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. *Veter- Res. Commun.* **2014**, *38*, 39–49, doi:10.1007/s11259-013-9583-9.
31. Tuo, Y.; Yu, H.; Ai, L.; Wu, Z.; Guo, B.; Chen, W. Aggregation and adhesion properties of 22 *Lactobacillus strains*. *J. Dairy Sci.* **2013**, *96*, 4252–4257, doi:10.3168/jds.2013-6547.
32. Reggi, S.; Giromini, C.; Dell'Anno, M.; Baldi, A.; Rebucci, R.; Rossi, L. *In Vitro* Digestion of Chestnut and Quebracho Tannin Extracts: Antimicrobial Effect, Antioxidant Capacity and Cytomodulatory Activity in Swine Intestinal IPEC-J2 Cells. *Animals* **2020**, *10*, 195, doi:10.3390/ani10020195.
33. Vergauwen, H.; Tambuyzer, B.; Jennes, K.; DeGroote, J.; Wang, W.; De Smet, S.; Michiels, J.; Van Ginneken, C. Trolox and Ascorbic Acid Reduce Direct and Indirect Oxidative Stress in the IPEC-J2 Cells, an *In Vitro* Model for the Porcine Gastrointestinal Tract. *PLoS ONE* **2015**, *10*, e0120485, doi:10.1371/journal.pone.0120485.
34. Brosnahan, A.J.; Brown, D.R. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. *Veter Microbiol.* **2012**, *156*, 229–237, doi:10.1016/j.vetmic.2011.10.017.
35. Tallon, R.; Arias, S.; Bressollier, P.; Urdaci, M. Strain-and matrix-dependent adhesion of *Lactobacillus plantarum* is mediated by proteinaceous bacterial compounds. *J. Appl. Microbiol*. **2007**, *102*, 442–451.
36. Lebeer, S.; Vanderleyden, J.; De Keersmaecker, S.C. Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. *Nat. Rev. Genet.* **2010**, *8*, 171–184, doi:10.1038/nrmicro2297.

37. Lähteinen, T.; Malinen, E.; Koort, J.M.; Mertaniemi-Hannus, U.; Hankimo, T.; Karikoski, N.; Pakkanen, S.; Laine, H.; Sillanpää, H.; Söderholm, H. Probiotic properties of Lactobacillus isolates originating from porcine intestine and feces. *Anaerobe* **2010**, *16*, 293–300.
38. Hsueh, H.-Y.; Yueh, P.-Y.; Yu, B.; Zhao, X.; Liu, J.-R. Expression of Lactobacillus reuteri Pg4 Collagen-Binding Protein Gene in *Lactobacillus casei* ATCC 393 Increases Its Adhesion Ability to Caco-2 Cells. *J. Agric. Food Chem.* **2010**, *58*, 12182–12191, doi:10.1021/jf1035756.
39. Minelli, E.B.; Benini, A.; Marzotto, M.; Sbarbati, A.; Ruzzenente, O.; Ferrario, R.; Hendriks, H.; Dellaglio, F. Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. *Int. Dairy J.* **2004**, *14*, 723–736, doi:10.1016/j.idairyj.2004.01.007.
40. Guo, Z.; Wang, J.; Yan, L.; Chen, W.; Liu, X.-M.; Zhang, H.-P. *In vitro* comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. *LWT* **2009**, *42*, 1640–1646, doi:10.1016/j.lwt.2009.05.025.
41. Wang, G.; Zhang, M.; Zhao, J.; Xia, Y.; Lai, P.F.-H.; Ai, L. A surface protein from *Lactobacillus plantarum* increases the adhe-sion of *lactobacillus strains* to human epithelial cells. *Front. Microbiol*. **2018**, *9*, 2858.
42. Hynönen, U.; Palva, A. Lactobacillus surface layer proteins: Structure, function and applications. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 5225–5243, doi:10.1007/s00253-013-4962-2.
43. Siezen, R.; Boekhorst, J.; Muscariello, L.; Molenaar, D.; Renckens, B.; Kleerebezem, M. *Lactobacillus plantarum* gene clusters encoding putative cell-surface protein complexes for carbohydrate utilization are conserved in specific gram-positive bacteria. *BMC Genom*. **2006**, *7*, 1–13.
44. Pretzer, G.; Snel, J.; Molenaar, D.; Wiersma, A.; Bron, P.A.; Lambert, J.; de Vos, W.M.; van der Meer, R.; Smits, M.A.; Kleer-ebezem, M. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *J. Bacteriol*. **2005**, *187*, 6128–6136.
45. Jensen, H.; Roos, S.; Jonsson, H.; Rud, I.; Grimmer, S.; Van Pijkeren, J.-P.; Britton, R.A.; Axelsson, L. Role of Lactobacillus reuteri cell and mucus-binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*. *Microbiology* **2014**, *160*, 671–681, doi:10.1099/mic.0.073551-0.
46. Wang, B.; Wei, H.; Yuan, J.; Li, Q.; Li, Y.; Li, N.; Li, J. Identification of a Surface Protein from Lactobacillus reuteri JCM1081 That Adheres to Porcine Gastric Mucin and Human Enterocyte-Like HT-29 Cells. *Curr. Microbiol.* **2008**, *57*, 33–38, doi:10.1007/s00284-008-9148-2.

4 *In vivo* evaluation of innovative functional feed additives



In vitro models are very important for preliminary evaluation of functional compounds. Moreover, the translation of results obtained from *in vitro* studies to *in vivo* is still not directly possible. The use of animals for scientific purposes is frequently debated in our society. The similarity between animals and humans, in particular for mammals, encouraged scientists to evaluate mechanisms and therapies in animal models before assessing them in humans. Humans and animals are complex organisms in which organs are involved in dense networks of hormones, cells crosstalk and circulating molecules. All levels of investigations, from healthy to diseased conditions, are necessary to understand biological mechanisms. *In vitro* models do not allow to study the interaction between organs, such as hormonal regulation and microorganisms' dissemination during infections. In these cases, *in vitro* studies cannot fully replicate the following connections, thus animals are still necessary. However, *in vitro* studies are still useful for formulating many hypotheses, that must be successively tested and validated *in vivo*. Still the most advanced cell cultures or separated tissues and organs technologies are far from being able to predict organisms functioning. Animals are not only similar from a biological point of view, but they also share human diseases with similar patterns. As example, Type I diabetes, hypertension, allergies, cancers, epilepsy are so similar that 90% of veterinary medications are identical or very close to those used for human medicine (Barré-Sinoussi and Montagutelli, 2015). Animals must not be used in case of other alternative non-animal-based model, with similar reliability, is available (Dir. 2010/63/EU). Research on relevant, carefully designed, well-characterized and controlled animal modes will remain for a long time an essential step for fundamental discoveries. Animal models have to be constantly improved to become more reliable and informative. However, animal ethic requires a permanent consideration and even if animal models seem to be antagonistic to ethics, they must be anchored for a high-quality science.

After the encouraging results achieved through *in vitro* studies, functional feed additives were also assessed for their effect *in vivo* on animal health and performance.

References

Barré-Sinoussi, F., Montagutelli, X., 2015. Animal models are essential to biological research: issues and perspectives. Future science OA 1.

Dir. 2010/63/EU, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance.

animals

MDPI

Article

Evaluation of Dietary Administration of Chestnut and Quebracho Tannins on Growth, Serum Metabolites and Fecal Parameters of Weaned Piglets

Valentina Caprarulo [1], Monika Hejna [1,2,*], Carlotta Giromini [1], Yanhong Liu [2], Matteo Dell'Anno [1], Stefania Sotira [1], Serena Reggi [1], Carlo Angelo Sgoifo-Rossi [1], Maria Luisa Callegari [3] and Luciana Rossi [1]

Brief Introduction of the Study:

Based on the encouraging results observed *in vitro*, the following paper evaluated the combination of chestnut and quebracho tannin extracts on animal performance, serum metabolites and faecal parameters in post-weaning piglets. The novelty of the study relies on the assessment of the inclusion of 1.25% of tannin extracts that did not impair the diet digestibility. The dietary supplementation of innovative additive significantly increased albumin, globulin, and decreased urea and creatinine. The *in vitro* test on swine intestinal cell line allowed to demonstrate the hormesis of tannins on cell viability and the possibility to enhance cell metabolism when subjected to oxidative stressor. These findings suggests that 1.25% of tannin extracts did not modify animal growth. Based on the observed results on cell viability the effect of tannins could be better exacerbated under stressful conditions.

4.1 Evaluation of Dietary Administration of Chestnut and Quebracho Tannins on Growth, Serum Metabolites and Fecal Parameters of Weaned Piglets

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Simple Summary: In pig livestock, alternatives to in-feed antibiotics are needed to promote health status and to control enteric infections. Plant extracts containing biologically-active molecules may influence animal metabolism and performance, therefore potentially reducing the use of antibiotics in pigs. Tannins have antioxidant, anti-inflammatory and antimicrobial properties, and have been adopted to enhance growth performance, modulate intestinal microbiota, and decrease the incidence of diarrhea, particularly during the post-weaning period. Despite their functional properties, tannins are known to be astringent compounds due to their ability to complex and precipitate proteins, particularly proline-rich proteins. The common use of non-standardized plant extracts generates results that are often controversial and difficult to interpret. In this study, attention was focused on the evaluation of the inclusion of a mixture of tannins extracted from chestnut and quebracho in the diet of piglets.

Abstract: In pig livestock, alternatives to in-feed antibiotics are needed to control enteric infections. Plant extracts such as tannins can represent an alternative as a natural source of functional compounds. The aim of this study was to evaluate the *in vitro* digestibility and *in vivo* effects of oral supplementation of combined chestnut (Ch) and quebracho (Qu) tannins in order to establish if they can induce a positive effect on weaned piglets' performance, metabolic status and fecal parameters. *In vitro* digestibility (dry matter, DM) of diets was calculated using a multi-step enzymatic technique. *In vitro* digested diet samples were further tested on an intestinal porcine enterocyte cell line (IPEC-J2). Weaned piglets (n = 120; 28 ± 2 day old) were randomly allotted to two groups (12 pens in total with 10 pigs per pen): control (Ctrl) and treatment (Ch/Qu). After one week of adaptation (day 0), 35-day-old piglets in the Ctrl group were fed a Ctrl diet and the Ch/Qu group were fed with 1.25% Ch/Qu for 40 days. Body weight and feed intake per pen were recorded weekly. At day 40, blood and fecal samples were collected. Principal metabolic parameters were evaluated from blood samples by enzymatic colorimetric analysis. Total phenolic compounds, urea, and ammonia in feces were analyzed (Megazyme International, Bray, Ireland). *In vitro* digestibility and cell viability assays showed that the inclusion of 1.25% Ch/Qu slightly reduced diet digestibility compared with the Ctrl diet, while intestinal cell viability was not altered with low concentrations of Ch/Qu digesta compared with Ctrl. *In vivo* results did not show any adverse effects of Ch/Qu on feed intake and growth performance, confirming that dietary inclusion of Ch/Qu at a concentration of 1.25% did not impair animal performance. The decreased diet DM digestibility in the Ch/Qu diet may cause increased serum concentration of albumin (Ctrl: 19.30 ± 0.88; Ch/Qu: 23.05 ± 0.88) and albumin/globulin ratio (Ctrl: 0.58 ± 0.04; Ch/Qu: 0.82 ± 0.04), but

decreased creatinine (Ctrl: 78.92 ± 4.18; Ch/Qu: 54.82 ± 4.18) and urea (Ctrl: 2.18 ± 0.19; Ch/Qu: 0.95 ± 0.19) compared with Ctrl. Pigs in the Ch/Qu group contained higher ($p < 0.05$) concentrations of fecal phenolic compounds and nitrogen than the Ctrl group, while fecal ammonia and urea were not affected by tannins. In conclusion, Ch/Qu tannin supplementation did not influence growth performance. Although lower digestibility was observed in the diet supplemented with Ch/Qu tannins, Ch/Qu supplementation did not show any adverse effect on intestinal epithelial cell viability.

Keywords: tannins; plant extracts; weaned piglets; zootechnical performances; blood metabolites; fecal nitrogen concentration

1. **Introduction**

In swine production, weaning is recognized as the most critical phase because piglets are exposed to various biological stressors, including physiological, environmental, and social changes, which lead to increases in the exposure to pathogens and dietary or environmental antigens [1–3]. For these reasons, antibiotics are often used to control bacterial infections and post-weaning diarrhea.

Livestock antibiotic resistance is a major global threat of increasing concern for animal and human health, which has thus led the European Union to ban the use of antibiotics as growth-promoting agents [4,5]. As a result, in the last decade, an increased use of zinc oxide at a pharmacological level (ZnO, 2000-3000 ppm) was observed as an alternative to antibiotics [6]. However, the widespread use of pharmacological levels of ZnO has raised concerns related to environmental issues and the potential increase in the prevalence of antibiotic resistant bacteria [4,7–9]. The Agency's Committee for Medicinal Products for Veterinary Use (CVMP) recommended the withdrawal of the existing marketing authorizations for veterinary medicinal products containing zinc oxide (EMA/394961/2017) [10].

Due to such restrictions, alternatives to antibiotics and alternatives to zinc oxide are urgently needed to guarantee animal production in line with health principles [11,12]. In this scenario, plant extracts can represent a valuable alternative as a natural source of functional compounds, such as polyphenolic compounds [13–17]. Among polyphenols, tannins derived from plant extracts are the largest class which can be classified into hydrolysable (HTs) or condensed (CTs) subgroups [18]. Tannins have been tested in the poultry and swine sectors, initially as tannin-rich feedstuffs (such as sorghum, barley, maize and fava beans), and more recently as tannin extracts from different plants (grape seed, grape pomace, acorns, oak, green tea leaves, and pomegranate) [19,20]. Chestnut trees (Ch, *Castanea sativa* Mill.) are a source of HTs, whereas quebracho trees (Qu, *Schinopsis* spp.) are a source of CTs or

proanthocyanidins [21]. Tannins extracted from these plants have been applied in intensive swine farms due to their antioxidant, anti-inflammatory, and antibacterial activities [16,21].

Heterogeneous results were obtained on the effects of Ch and Qu to enhance growth performance, modulate intestinal microbiota, and decrease the incidence of diarrhea during the post-weaning period [22–24]. These heterogeneous results could be related to the chemical characteristics of tannins, which can compromise the palatability, digestibility, and protein use of feed. The ability to bind proteins and carbohydrates in monogastric animals is associated with the antinutritional effects of tannins in reducing feed palatability [18]. Thus, contrasting results on the effective supplementation of tannins on pigs' performance and intestinal health have been observed in relation to the source of tannins (Ch and Qu), dosage of tannins, and the type of tannins (HTs or CTs) included in the diets [25,26]. Therefore, the heterogeneity of commercial products is associated with the use of Ch or Qu individually or in mixtures with different percentages of tannins (from 54% to 82%) and hence different amounts of HTs and CTs.

However, the studies presented in the literature for pigs in which Ch and Qu has been used showed that most of the products mainly contained tannins extracted from Ch (a source of HTs) [22,25,26]; no studies that adopted the use of Qu tannins (a source of CTs) have been reported and only a few studies that adopted the combination of Ch and Qu tannins (source of HTs/CTs) have been published [26–29]. Further research to fully understand the synergistic effect of HTs/CTs, derived from both chestnut and quebracho, on the growth performance of weaned piglets is needed.

In light of this, identifying the correct application dose is essential in order to maximize the beneficial effects of tannins, and minimize the antinutritional effects on animal growth performance and health. Thus, the main purpose of this study was to evaluate the *in vitro* dry matter (DM) digestibility and *in vivo* effect of Ch/Qu in order to establish if the inclusion of 1.25% combined chestnut and quebracho tannins can induce a positive effect on weaned piglets.

2. Materials and Methods

2.1. *Animals, Housing, Experimental Design and Treatment*

Our *in vivo* trial complied with Italian regulations on animal experimentation and ethics (DL 26/2014) [30] in accordance with European regulation (Dir. 2010/6) [31] and was approved by the Animal Welfare Body of the University of Milan (number 31/2019). This trial was performed in an intensive conventional herd farm located in Lombardy (Italy) that was free from any of the diseases reported in the ex A-list of the

International Office of Epizootics, and free from Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome, and salmonellosis.

A total of 120 crossbred piglets (Large White × Landrace), weaned at 28 ± 2 days (50% female and 50% male), were identified using individual ear tags and allotted in randomized complete block design into two experimental groups: control group (Ctrl) and treatment group (Ch/Qu). There were 60 pigs per treatment with 6 replicate pens and 10 pigs per pen. The groups were homogeneous in terms of gender, weight and litter. After one week of adaptation (considered day 0, piglets were 35 days old), during which all animals received the same basal diet, the experimental diets were distributed ad libitum to all animals for 40 days. Experimental diets (Plurimix, Fabermatica, CR, Italy) were formulated according to animal requirements for the post-weaning phase (Ferraroni Mangimi SpA, Bonemerse, Italy). The two diets were isoenergetic and isoproteic and fulfilled the NRC (2012) [32] requirements for post-weaned piglets (Table 1). The Ch/Qu diet was differentiable by the inclusion of 1.25% of tannin extract from chestnut and quebracho trees (Silvafeed for Swine, Silvateam, Italy) (Table 1).

Table 1. Ingredient composition of the experimental diets administered to weaned piglets (control (Ctrl), $n = 60$; chestnut/quebracho (Ch/Qu), $n = 60$) from day 0 to day 40 of the experimental trial on an as-fed basis.

Ingredients [1,2,3,4,5], as % of Fed Basis	**Ctrl**	**Ch/Qu**
Barley meal	25.15	25.00
Wheat meal	19.41	19.07
Corn meal	14.03	13.50
Corn flakes	4.85	4.80
Soybean meal	4.65	4.60
Soybean protein	4.11	4.10
Bakery meal	4.00	4.00
Dextrose monohydrate	3.50	3.50
Wheat middlings	4.32	4.30
Fermented milk product	3.00	3.00
Fish meal	2.50	2.50
Milk whey powder	2.50	2.50
Coconut oil	1.00	1.00
Soy oil	1.00	1.00
Plasma, meal	1.00	1.00
Dicalcium phosphate	0.85	0.80
Animal fats, lard	0.70	0.70
L-Lysine	0.50	0.50
Acidity regulators[5]	1.00	1.00
Benzoic acid	0.40	0.40
L-Threonine	0.34	0.34
DL-Methionine	0.35	0.35
Sodium chloride	0.26	0.24

Vitamins	0.24	0.24
L-Valine (96.5%)	0.14	0.14
L-Tryptophan	0.08	0.05
Copper sulfate	0.04	0.04
Ch/Qu Tannins [6]	-	1.25

[1]Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%). [2] Nutrient and digestible energy content was calculated using Plurimix software (Fabermatica, CR, Italy). [3] Nutrient and digestible energy content (expressed the as-fed basis) of diet: dry matter (DM), 89.37%; crude protein, 16.92%; crude Fat, 5.06%; crude fiber, 3.15%; DE, 3.43 Mc/Kg. DE = digestible energy content estimated from NRC (2012). [4] Supplied the following nutrients per kg of diet: 10,000 UI vitamin A, 1000 UI vitamin D3, 100 mg UI vitamin E, 3 mg vitamin B1, 96.3 mg vitamin B2, 5.8 mg vitamin B6, 27 mg vitamin B5, 0.040 mg vitamin B12, 4.8 mg vitamin K3, 0.19 mg biotin, 35 mg niacinamide, 1.4 mg folic acid, 120 mg choline chloride, 70 mg betaine chloride, 108 mg Fe as $FeCO_3$, 38.5 mg Mn as MnO_2, 112 mg Zn as ZnO, 19.3 Cu as $CuSO_4$, 0.58 I as $Ca(IO_3)_2$, 0.29 Se as Na_2SeO_3. [5] Organic Acids: formic acid, sodium formate, sorbic acid, orthophosphoric acid, calcium formate, citric acid, and fumaric acid; [6] Commercial chestnut and quebracho tannin extract (Silvafeed Nutri P/ENC for Swine, Silvateam, Italy).

The enrolled piglets of both experimental groups were reared in one unique room at constant temperature (27 °C) and humidity (60%) for the entire experimental period. The room had an unobstructed floor area available to each weaner piglet of 0.40 m², according to Directive 2008/120/EC [33]. Each pen was equipped with nipple drinkers with ad libitum access to fresh water.

2.2. Chemical Analyses and in vitro Digestibility Evaluation of Ctrl and Ch/Qu Diets

Feed samples from Ctrl and Ch/Qu (500 g each) were collected in order to guarantee the representativeness of samples according to the Reg. 152/2009/EC [34] and ISO 24333:2009 [35]. Moreover, a total of 50 g of commercial product supplemented in the Ch/Qu diet (Silvafeed Nutri P/ENC for Swine, Silvateam, Italy) was collected. The Ctrl and Ch/Qu diets as well as the tannin supplements were analyzed for proximate analysis, including moisture, crude protein (CP), crude fibre (CF), ether extract (EE), and crude ash [36]. Specifically, moisture determination was performed by oven-drying at 135 °C for 2 h. Crude protein content was measured according to the Kjeldahl method. Crude fiber was determined by the Filter Bag technique. Ether extract content was determined by the Soxhlet method with prior hydrolysis. Ash was measured using a muffle furnace at 550 °C. Fecal samples were also analyzed for moisture following the procedure described above.

Total phenolic compounds in Ctrl and Ch/Qu diets and in Ch/Qu tannin extracts were assayed according to the Folin-Ciocalteu method [37]. Each feed sample was weighed (5 ± 0.5 g) and mixed with 30 mL of pure methanol (Sigma Chemical Co, St. Louis, MO, USA) for 24 h at room temperature and subsequently filtered (Whatman 54, Florham Park, NJ, USA). The obtained chemical extracts from feed samples were tested for total phenolic compounds. Prior to the analysis, a standard curve was

prepared using tannic acid (Sigma Chemical Co, St. Louis, MO, USA). The tannic acid was water-dissolved to obtain a stock solution of 960 μg/mL. Dilutions of the stock solution were prepared to obtain final concentrations from 60 to 960 μg tannic acid/mL. The Folin-Ciocalteu reagent was diluted 1:10 with deionized water and a solution of 1 M sodium carbonate (Sigma Chemical Co, St. Louis, MO, USA) was prepared. Briefly, an aliquot (0.5 mL) of extract, blank or standard was placed in a 15 mL plastic tube, where the Folin-Ciocalteu reagent (2.5 mL) and sodium carbonate (2 mL) were added and the mixture was incubated at room temperature in a dark chamber for 20 min. The total phenolic content was determined by colorimetry at 630 nm using an UV-visible spectrophotometer (Jasco V-630, Easton, MD, USA). Total phenolic content was expressed as tannic acid equivalents (g TAE/kg). The analyses were performed in technical duplicate and biological triplicate.

The Ctrl and Ch/Qu diets adopted in the *in vivo* trial were *in vitro* digested using the protocol reported by Reggi et al. [16]. The *in vitro* digestion was performed according to the protocol described by Regmi et al. [38] with minor adaptations previously reported by our group [39]. At the end of the *in vitro* digestion procedure, a soluble fraction and an undigested fraction (UF) were obtained. The soluble fraction was used for cell viability assays (detailed below). The UF was then collected in a filtration unit using a porcelain filtration funnel lined with pre-weighed filter paper (Whatman no. 54). The UF, along with the filter paper, were dried overnight at 65 °C. The UF was used to calculate the *in vitro* digestibility (IVD) using the following equation:

$$\text{IVD (\%)} = (\text{sample (DM)} - \text{sample UF (DM)})/(\text{sample (DM)} \times 100).$$

The digestion procedure was performed twice (2 biological replicates). Whey protein (90%) was included as a reference sample for stability tests in all digestions performed, as previously indicated in Giromini et al. [17].

2.3. Effect of Ctrl and Ch/Qu Diet on Swine Intestinal Cell Viability

The intestinal porcine enterocyte cell line IPEC-J2 is unique as it is derived from the small intestine isolated from the jejunum of a neonatal unsuckled piglet (ACC 701, DSMZ, Braunschweig, Germany) and is not transformed nor tumorigenic in nature [39]. IPEC-J2 cells were cultured in Dulbecco's Modified Eagle Medium + Ham's F-12 mixture (DMEM/F-12) supplemented with HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)), fetal bovine serum (FBS), penicillin/ streptomycin and cultivated in a humid chamber at 37 °C with 5% CO_2. All experiments were performed using IPEC-J2 cells within six cell passages (passages 16 to 22) to ensure

reproducibility. In particular, IPEC-J2 cells were seeded at a density of 1.5-2 × 10^5 cells/mL in 96-well plates and cultured for 24 h.

Samples of *in vitro* digested Ctrl and Ch/Qu diets (soluble fraction of the *in vitro* digestion described above) were used to obtain a dose-response curve in IPEC-J2 cells. Diluted concentrations of digesta were applied to cells (21.31-0.33 mg/mL), while DMEM/F-12 mix alone was used as a negative control (0 mg/mL, DMEM). Cell viability was determined after a three-hour incubation by a colorimetric proliferation assay (MTT test, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) in accordance with the manufacturer's instructions (Sigma Chemical Co, St. Louis, MO, USA).

2.4. Collection of Fecal and Blood Samples

Fecal (n = 6) and serum blood samples (n = 12) were collected at day 40 of the *in vivo* trial (from 75-day-old piglets), according to ethical authorization, from a randomly selected subset of animals (blood: n = 12/treatment fecal: n = 6/treatment, 50% female and 50% male) for each treatment group cohort one hour before the morning feeding and within one hour after feeding in order to have homogeneous conditions and representative parameters. Fecal samples were individually collected from rectal ampulla and immediately stored at −20 °C until further analysis. From each piglet included in the subset, blood was collected from the jugular vein into vacuum tubes, maintained for 2 h at room temperature, and then centrifuged at 850 r.c.f. (relative centrifugal force) for 10 min at 4 °C. Serum was aliquot and stored at −20 °C for further analysis.

2.5. Zootechnical Evaluation

Piglets were individually weighed (BW) on day 0, 14, 28 and 40 of the *in vivo* trial (35-, 49-, 63- and 75-day-old piglets, respectively). The amount of feed offered ad libitum to the experimental groups (Ctrl and Ch/Qu) was recorded. The feed intake of individual pen (experimental unit for the feed intake evaluation) was calculated every week by measuring the total refusals. The average daily feed intake (ADFI) and feed-to-gain ratio were calculated from day 0 to 14, from day 14 to 28 and from day 28 to 40 of the *in vivo* trial (35–49-, 49–63- and 63–75-day-old piglets, respectively). Based on the ADFI and total phenolic compounds in the feed, phenolic compound intake was calculated from day 0 to 14, from day 14 to 28 and from day 28 to 40 of *in vivo* trial (35–49-, 49–63- and 63–75-day-old piglets, respectively). The health status of the piglets was monitored daily. Mortality was registered, and the incidence of diarrhea was calculated based on the number of piglets with clinical sign of diarrhea [11].

2.6. Total Phenolic Compounds, Urea and Ammonia in Feces

The total phenolic compounds in feces were evaluated as previously described. Fecal samples were weighed (5 ± 0.5 g) and mixed with 30 mL of pure methanol (Sigma Chemical Co, St. Louis, MO, USA) for 24 h at room temperature and subsequently filtered (Whatman 54, Florham Park, NJ, USA). The obtained chemical extracts from fecal samples were tested for total phenolic compounds according to the Folin-Ciocalteu method [37].

Fecal samples (5 g) were treated prior to analysis with 20 mL of perchloric acid (1 M). Then, samples were homogenized for 2 min using an Ultra-turrax (T25, Ika Works Inc., Wilmington, NC, USA). The homogenized samples were adjusted to pH 8 with KOH (2 M) and adjusted to the mark with 100 mL of distilled water. The samples were then maintained on ice for 20 min and centrifuged at 13,000 × *g* for 10 min. The supernatant was filtered (Whatman 1, Florham Park, NJ, USA). A K-URAMR test kit (Megazyme, Bray, Ireland) was used for urea and ammonia analysis. The test kit method involved urease, which catalyzed the hydrolysis of urea to ammonia and the subsequent reaction of ammonia, 2-oxoglutarate and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) in the presence of glutamate dehydrogenase to form glutamic acid and NADP+. The consumption of NADPH was measured by the decrease in absorbance at 340 nm using a UV-visible spectrophotometer (Jasco V-630, Easton, MD, USA) and was proportional to the original amount of urea over a finite range (Urea/Ammonia (Rapid) Assay Procedure K-URAMR 11/05, Megazyme International, Bray, Ireland). The analyses were performed in technical duplicate and biological triplicate.

2.7. Blood Serum Analysis

Serum biochemical analyses were performed by the Lombardy and Emilia Romagna Experimental Zootechnic Institute (IZSLER). The concentration of total protein (g/L), albumin (g/L), globulin (g/L), albumin/globulin (A/G ratio), alanine aminotransferase (ALT-GPT; IU/L), aspartate aminotransferase (AST-GOT; IU/L), alkaline phosphatase (ALP; IU/L), glucose (mmol/L), urea (mmol/L), creatinine (μmol/L), total bilirubin (umol/L), total cholesterol (mmol/L), triglycerides (mmol/L), high-density lipoprotein (HDL; mmol/L), low-density lipoprotein (LDL; mmol/L), calcium (mmol/L), phosphorus (mmol/L), and magnesium (mmol/L) were measured. The parameters were analyzed at 37 °C via standard enzymatic colorimetric analysis using a multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA).

2.8. Statistical Analysis

One-way ANOVA was calculated using SAS 9.4 (SAS Inst. Inc., Cary, NC, USA) and was used to analyze digestibility and cell viability data. Animal performance, phenolic compounds (ingestion and diet, supplement and fecal content), fecal protein, nitrogen, ammonia, urea and blood metabolite data were analyzed using a generalized linear mixed model through generalized linear mixed model Proc GLIMMIX SAS 9.4 (SAS Inst. Inc., Cary, NC, USA) [40]. For animal performance, the model included the fixed effect of treatments (Trt), experimental day (Day) and the interaction between the two factors (Trt × Day), and the repeated measures over time were included in the RANDOM statement. Tukey-Kramer studentized adjustments were used to separate treatment means within the two-way interactions. Within significant two-way interactions, the slice option was used to separate means within specific treatments and experimental days. The proc CORR procedure was used to calculate and test Spearman correlations by treatment among feed (ADFI, phenolic compound intake), fecal (fecal phenolic compounds, protein, nitrogen, ammonia, urea) and blood metabolites (glucose, urea, total cholesterol, HDL, LDL, triglycerides) on day 40. Means were considered different when $p \leq 0.05$. Results are reported as least squares means (LSMEANS) and standard errors of the means (SEM).

3. Results

3.1. Chemical Analyses and in vitro Digestibility Evaluation of Experimental Ch/Qu and Ctrl Diets

The experimental diets contained a similar content of principal nutrients (Table 2). The phenolic content differed due to the tannin supplementation, and was 3.68 times higher in Ch/Qu group than in the Ctrl group. The *in vitro* experiments showed a slight reduction of DM digestibility in the Ch/Qu diet (69.33% of DM) compared to the Ctrl diet (72.00% of DM).

Table 2. Chemical analysis of experimental diets fed to piglets from day 0 to day 40 [1] of the *in vivo* trial (from 35- to 75-day-old piglets).

Principal nutrients	**Experimental Diets** [1]		**Tannin Additive**
	Ctrl	**Ch/Qu**	**Tannins** [2]
Moisture, %	9.54	9.66	6.28
Crude protein, %	17.40	17.72	3.06
Crude fiber, %	3.27	3.45	nd [3]
Crude fat, %	4.47	4.94	nd [3]
Ash, %	5.32	5.71	1.29
Phenolic compound, g TAE/kg	0.79 ± 0.03	2.91 ± 0.06	715.05 ± 51.02

[1] Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%). [2] Commercial hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Silvateam, Italy). [3] nd = not detectable.
g TAE/kg: tannic acid equivalents.

3.2. Swine Intestinal Cell Viability

Samples of *in vitro* digested Ch/Qu and control diets were tested on IPEC-J2 cell viability at diluted concentrations. In general, Ch/Qu and Ctrl digesta showed comparable effects on cell viability at the tested concentrations. An exception was represented by the concentration of 5.32 mg/mL of digesta. At this concentration, Ch/Qu seems to detrimentally affect viability compared to Ctrl. In contrast, at a concentration of 1.33 mg/mL, Ch/Qu promoted cell viability compared to Ctrl ($p < 0.05$) (Figure 1).

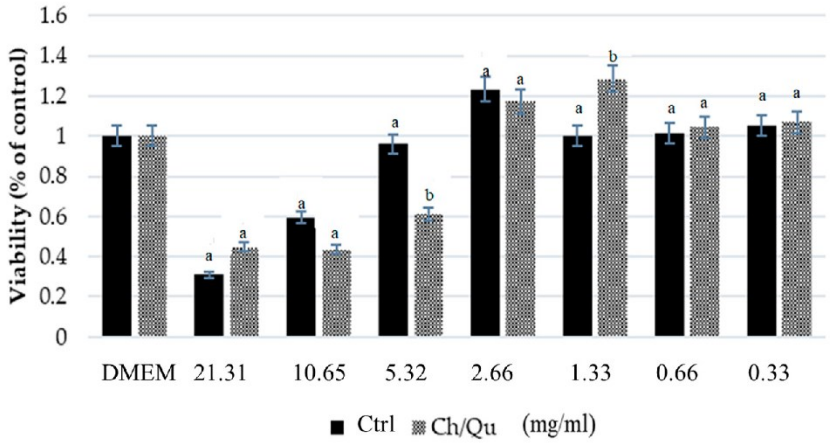

Figure 1. Effect of different concentrations (21.31–0.33 mg/mL) of Ctrl and Ch/Qu digesta (<3 kDa) on intestinal porcine epithelial cell line IPEC-J2 cell viability (via 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide MTT assay). Data are expressed as percentage of control (DMEM - Dulbecco's Modified Eagle Medium + Ham's F-12 mixture, 0 mg/mL) as least squares means (LSMEANS) and standard errors of the means SEM (n = 3). At each concentration, different letters denote statistical differences between Ch/Qu and Ctrl.

3.3. Zootechnical Performance

No mortality and no veterinary interventions were observed in the Ch/Qu and Ctrl groups during the entire experimental period. Moreover, no differences between males and females were observed in the analyzed parameters. Tannin supplementation did not affect ADFI, BW, ADG or feed-to-gain ratio (Table 3). Daily phenolic compound intake was significantly higher ($p < 0.01$) in the Ch/Qu group compared to the Ctrl group during the entire experimental period.

Table 3. Growth performance of weaned piglets fed diets with tannins (Ch/Qu, n = 60) or without supplementation (Ctrl, n = 60) from day 0 to day 40 [1] of the *in vivo* trial (from 35- to 75-day-old piglets).

Growth performances	Treatments [2]	SEM	*p*-Value

	Ctrl	Ch/Qu		Trt	Day	Trt × Day
Phenolic compound intake, g/d	0.656 [a]	2.341 [b]	0.15	<0.01	<0.01	<0.01
ADFI, kg/d	0.623	0.599	0.02	0.258	<0.01	0.241
BW, kg	13.86	13.64	0.64	0.819	<0.01	0.987
ADG, kg/d	0.292	0.284	0.02	0.729	<0.01	0.651
Feed:Gain, d	1.98	2.04	0.07	0.510	0.040	0.140

[a,b] Indicate differences between treatment groups at $p < 0.05$ within the same day (Trt × Day: $p < 0.05$). [1] Data are shown as LSMEANS and SEM. [2] Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%). ADFI: average daily feed intake; BW: body weight; ADG: average daily gain.

Regarding the health status of the animals throughout the experimental period in both groups, clinical signs of diarrhea occurred after seven days and the signs were transient (on average lasting for three days). The occurrence of diarrhea (number of new animals with signs of diarrhea per group) was 3.39% and 5.00% in the Ctrl and Ch/Qu groups at day 0-14, respectively; at day 14-28, the incidence was 18.64% and 15.52% in the Ctrl and Ch/Qu groups; and at day 28-40, the incidence was 1.69% and 3.45% in the Ctrl and Ch/Qu groups (Supplementary Table 1).

3.4. Blood Serum Metabolites

Dietary treatment with tannins did not influence total protein content, globulin, ALT-GPT, AST-GOT, ALP, glucose, total bilirubin, total cholesterol, triglycerides, HDL, LDL, calcium, phosphorus or magnesium (Table 4). Pigs in the Ch/Qu group had higher albumin ($p = 0.006$) and A/G ratio ($p = 0.001$), but lower creatinine ($p < 0.001$) and urea ($p = 0.001$) compared with pigs in the Ctrl group.

Table 4. The biochemical concentration of serum metabolites in control (Ctrl, $n = 12$) and tannin (Ch/Qu, $n = 12$) groups on day 40 of the *in vivo* trial (75-day-old piglets) with reference ranges for pigs.

Blood [1]	Treatments [2]		SEM	*p*-Value	Reference Range
	Ctrl	Ch/Qu		Treatment	
Total protein content, g/L	52.88	52.18	1.89	0.798	44–74 [41–43]
Albumin, g/L	19.30 [a]	23.05 [b]	0.88	0.006	19–39 [42]
Globulin, g/L	33.58	29.80	1.55	0.053	19–41 [41,43]
A/G ratio	0.58 [a]	0.82 [b]	0.04	0.001	0.50–2.2 [41–43]
ALT-GPT, IU/L	38.33	33.82	2.51	0.218	36–117 [43]
AST-GOT, IU/L	54.17	48.09	3.41	0.221	21–98 [42]
ALP, UI/L	165.67	186.00	12.58	0.266	46–341 [43]
Glucose, mmol/L	5.00	5.05	0.25	0.878	3.5–8.6 [41]
Urea, mmol/L	2.18 [a]	0.95 [b]	0.19	< 0.001	0.90–8.89 [41,42]
Creatinine, µmol/L	78.92 [a]	54.82 [b]	4.18	0.001	67–172 [42]
Total bilirubin, umol/L	1.98	1.67	0.13	0.107	0.9–3.4 [42]
Total cholesterol, mmol/L	2.51	2.38	0.13	0.479	1.3–4.2 [41–43]
Triglycerides, mmol/L	0.68	0.58	0.06	0.259	0.3–2.7 [41]
HDL, mmol/L	0.77	0.75	0.03	0.667	-
LDL, mmol/L	1.60	1.52	0.10	0.532	-

Calcium, mmol/L	2.28	2.29	0.06	0.923	2.02–3.21[42]
Phosphorus, mmol/L	3.05	3.02	0.08	0.774	1.46–3.45 [42]
Magnesium, mmol/L	0.85	0.80	0.02	0.214	0.9–1.2 [41]

A/G = albumin/globulin; ALT-GPT = alanine aminotransferase; AST-GOT = aspartate aminotransferase; ALP = alkaline phosphatase; HDL = high-density lipoprotein; LDL = low density lipoprotein. [a,b] Indicates differences among treatment groups at $p < 0.05$ within the same day. [1] Data are shown as LSMEANS and SEM. [2] Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%).

3.5. Influence of Dietary Treatment with Tannins on Feces

Weaned piglets in the Ch/Qu group showed higher concentrations of fecal phenolic compounds than the Ctrl group ($p = 0.047$). Fecal nitrogen concentrations were significantly higher ($p = 0.002$) in the Ch/Qu group than the Ctrl group, while fecal ammonia ($p = 0.684$) and urea ($p = 0.235$) were not affected by the dietary inclusion of tannins (Table 5).

Table 5. Fecal metabolites of weaned piglets fed diets with tannins (Ch/Qu, $n = 6$) or without tannin supplementation (Ctrl, $n = 6$) on day 40 [1] of the *in vivo* trial (from 35- to 75-day-old piglets).

Feces	Treatments [2]		SEM	*p*-Value
	Ctrl	Ch/Qu		Treatment
Phenolic compound, g TAE/kg	0.34 [a]	0.50 [b]	0.05	0.047
Nitrogen, g/kg [3]	36.02 [a]	41.35 [b]	1.00	0.002
Ammonia, g/100g	2.26	3.20	1.89	0.684
Urea, g/100g	0.62	0.99	0.23	0.235

[a,b] Indicates differences among treatment groups at $p < 0.05$ within the same day. [1] Data are shown as least squares means LSMEANS and standard errors of the means SEM. [2] Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%). [3] Data are shown as fresh weight.
g TAE/kg: tannic acid equivalents.

3.6. Influence of Dietary Treatment with Tannins on Correlation among Feed, Feces and Blood Parameters

We found a significantly positive correlation between fecal nitrogen and phenolic compound intake ($p = 0.007$). There was also a negative correlation between fecal nitrogen and blood urea (Table 6).

Table 6. Correlation (Spearman correlation) among feed, feces ($n = 6$/treatment) and blood parameters ($n = 6$/treatment) on day 40 [1,2] of the *in vivo* trial (from 35- to 75-day-old piglets).

Feces	Parameters Header	Spearman r	*p*-Value
Fecal nitrogen, g/kg DM	Blood urea, mmol/L	−0.86	0.014
	Phenolic compound intake, g/d	0.89	0.007

[1]Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%). [2]Spearman correlation considering the experimental groups (Ctrl and Ch/Qu).

4. Discussion

The present study included two experimental sections. Firstly, an *in vitro* characterization of the Ch/Qu and Ctrl diets (chemical composition, *in vitro* digestibility (DM) and cell viability assays) was performed. Secondly, an *in vivo* experimental trial in swine was conducted during which the Ch/Qu and Ctrl diets were administered to animals.

The *in vitro* results showed that Ch/Qu *in vitro* digestibility was slightly but not significantly reduced compared with Ctrl diet digestibility, suggesting that the presence of Ch and Qu tannins in the diet may limit nutrient digestibility due to binding and forming stable and insoluble complexes with proteins [44,45].

In addition, results obtained in IPEC-J2 cells showed that the Ch/Qu diet had a similar effect on intestinal epithelial cell viability compared to the Ctrl diet. The tested concentration range allowed us to observe a hormetic effect in IPEC-J2 cell viability, as indicated by the fact that low concentrations were stimulatory while the high concentrations were harmful to cell viability [46]. Despite their antimicrobial and antioxidant properties, the supplementation of tannins in animal feed could decrease feed palatability and the absorption, digestion, and utilization of dietary proteins [18,47]. However, in our study, the feed intake was the same for the Ctrl and Ch/Qu groups; thus, the inclusion of 1.25% Ch/Qu tannins in the diet did not affect feed palatability. Moreover, growth performance of the Ctrl and Ch/Q groups did not show any significantly differences. In fact, BW and ADG remained at a similar level, confirming that the dietary inclusion of Ch/Qu did not impair animal performance due to the protein binding property of tannins [48].

Different literature cases have shown contradictory results relating to the dosages of tannin inclusion. In line with our study, the inclusion of 1%, 2% and 3% of Ch/Qu or Ch tannins had no effect on ADG, BW and feed efficiency in pigs [26,28], whereas Ch/Qu tannin supplementation at 2% showed a positive effect on ADFI and ADG [23]. Furthermore, Bee et al. [47] reported that the inclusion of 3% Ch/Qu tannins significantly decreased the gain-to-feed ratio in boars, while BW and ADG were not influenced. Moreover, lower doses (from 0.11% to 0.45%) of Ch tannins did not improve growth performance in piglets [22,27]. The contradictory literature results related to dosages could thus be related to different compositions of tannin-based commercial products. In light of this, the dosages of tannins cannot be a unique explanation for the effect on animal performance.

In fact, the combined effect of Ch/Qu tannins could be exacerbated during stressful conditions, such as experimental bacterial infections [24,28]. According to Reggi et al. [16], beneficial effects were reported when Ch/Qu digesta were administered to experimentally stressed intestinal swine cells, suggesting that it might have a trophic effect at the intestinal epithelium, and an increased viability of cells was

observed after tannin treatment. In present study, animals were reared in a conventional herd farm. The incidence of diarrhea was not different between the Ctrl and Ch/Qu groups during the entire experimental period. Moreover, we observed a decrease of diarrhea incidence in the Qu/Ch group from day 14 to 28 when compared to the Ctrl group, and the occurrence of diarrhea was below the average post-weaning level [49,50].

The physiology and hematological parameters of animals can be influenced by several factors, including nutrition [51]. In the present study, all biochemical parameters for both the Ctrl and Ch/Qu groups were within the reference range of weaned pigs [41–43]. The increase in serum albumin and A/G ratio in the Ch/Qu group compared to the Ctrl group were in line with results by Chedea et al. [52]. Albumin is an important indicator of protein status; the increment of this serum metabolite could be due to increased microbial protein in the intestine, which might trigger and increase the amount of amino acids [53]. The level of the blood A/G ratio could be related to protein synthesis and the humoral immunity of animals [54]. Our study revealed a significant decrease in serum creatinine and urea in the Ch/Qu group compared to the Ctrl group. Creatinine and urea are types of non-protein nitrogen related to protein catabolism [55]. The main source of creatinine in serum is associated with the degradation of creatine in animal muscle. Creatinine levels in the blood may increase when diarrhea occurs due to the increased mobilization of muscle protein to compensate for the lack of nutrient absorption. Thus, the decrement of serum creatinine could be associated with the reduction of nutrient availability. Despite the small decrement of digestibility in the Ch/Qu diet observed in the *in vitro* trial, this scenario can lead to decreased nutrient utilization (in particular, protein availability).

Moreover, we hypothesized that there would be a shift in mild protein metabolism principally associated with the protein binding properties of tannins causing a depression in digestive capacity in the small intestine, but we showed the opposite (signs of a growth promoting substance). Moreover, we can hypothesize that there was a shift on mild protein metabolism principally associated with the protein binding properties of tannins causing depression of digestive capacity in the small intestine and showed the opposite signs of a growth promoting substance. In fact, in the Ch/Qu group, we also detected a decrement in serum urea, another important indicator of protein status and of feed efficiency [56]. Thus, the decrement of blood urea concentration could be due to the increase of the synthesis of bacterial proteins [57,58]. Tannins promote bacterial growth in the large intestine, which are able to formulate undigested substrate. We found an increased fecal content of polyphenols and nitrogen concentration in the Ch/Qu group, which could be associated with the bio-accessibility and degradation of tannins in the intestinal tract [57,59].

Literature studies showed that proanthocyanidins were not completely absorbed in the gastrointestinal tract, resulting in a higher polyphenol content in the feces of pigs [59]. Additionally, several studies have shown that an increased concentration of fecal polyphenols and nitrogen may be due to the ability of tannins to form stable complexes with proteins [48,59]. In the present study, the positive correlation between phenolic compound intake and fecal N concentration could indicate that phenolic compounds may form protein-tannin complexes and could decrease protein digestibility [48,57,58]. The negative correlation between fecal nitrogen and blood urea could explicate our results. In fact, similar results were reported in the literature where a higher excretion of N through feces was associated with a reduction in blood urea concentration [60].

In light of this, we could hypothesize a possible shift in protein metabolism, which has led to the modulation of serum creatinine, serum urea concentration and fecal metabolites principally related to protein utilization and absorption in the Ch/Qu group.

5. Conclusions

Tannins are used mainly for their antimicrobial and antioxidant properties in animal nutrition. In our study, we observed a slight reduction of Ch/Qu diet digestibility and protein utilization, but no effects on feed intake and growth performance were observed. Moreover, *in vitro* results on IPEC-J2 cells showed that the presence of Ch/Qu tannins in the diet did not impair intestinal cell viability. Furthermore, Ch/Qu supplementation modulated serum creatinine and urea concentration, probably due to a modulation of the entire intestine digestive capacity, which also led to increased fecal nitrogen concentration.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/10/11/1945/s1, Table S1: The clinical signs of diarrhea of weaned piglets fed diets with tannins (Ch/Qu, n=60) or without (Ctrl, n=60) supplementation from day 0 to day 40 of *in vivo* trial.

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References

1. Rossi, L.; Vagni, S.; Polidori, C.; Alborali, G.L.; Baldi, A.; Dell'Orto, V. Experimental induction of *Escherichia coli* diarrhoea in weaned piglets. *J. Vet. Med.* **2012**, *2*, 1–8.
2. Rossi, L.; Di Giancamillo, A.; Reggi, S.; Domeneghini, C.; Baldi, A.; Sala, V.; Dell'Orto, V.; Coddens, A.; Cox, E.; Fogher, C. Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. *J. Vet. Sci.* **2013**, *14*, 263–270, doi:10.4142/jvs.2013.14.3.263.
3. Rossi, L.; Pinotti, L.; Agazzi, A.; Dell'Orto, V.; Baldi, A. Plant bioreactors for the antigenic hook-associated flgK protein expression. *Ital. J. Anim. Sci.* **2014**, *13*, 2939.
4. Rossi, L.; Dell'Orto, V.; Vagni, S.; Sala, V.; Reggi, S.; Baldi, A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. *Vet. Res. Commun.* **2014**, *38*, 39–49, doi:10.1007/s11259-013-9583-9.
5. EU Directive 1831/2003 of the European Parliament and of The Council on additives for use in animal nutrition. *Off. J. Eur. Union* **2003**. OJ L 268, 29-43.
6. Cromwell, G. Why and how antibiotics are used in swine production. *Anim. Biotechnol.* **2002**, *13*, 7–27, doi:10.1081/ABIO-120005767.
7. Hejna, M.; Gottardo, D.; Baldi, A.; Dell'Orto, V.; Cheli, F.; Zaninelli, M.; Rossi, L. Review: Nutritional ecology of heavy metals. *Animal* **2018**, *12*, 2156–2170, doi:10.1017/S175173111700355X.
8. Hejna, M.; Moscatelli, A.; Stroppa, N.; Onelli, E.; Pilu, S.; Baldi, A.; Rossi, L. Bioaccumulation of heavy metals from wastewater through a *Typha latifolia* and *Thelypteris palustris* phytoremediation system. *Chemosphere* **2020**, *241*, 125018.
9. Hejna, M.; Moscatelli, A.; Onelli, E.; Baldi, A.; Pilu, S.; Rossi, L. Evaluation of concentration of heavy metals in animal rearing system. *Ital. J. Anim. Sci.* **2019**, *18*, 1372–1384.
10. European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), & European Medicines Agency (EMA). ECDC/EFSA/EMA Second Joint Report on the Integrated Analysis of the Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Humans and Food-

Producing Animals: Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA J.* **2017**, *15*, e04872.
11. Sotira, S.; Dell'Anno, M.; Caprarulo, V.; Hejna, M.; Pirrone, F.; Callegari, M.L.; Tucci, T.V.; Rossi, L. Effects of tributyrin supplementation on growth performance, insulin, blood metabolites and gut microbiota in weaned piglets. *Animals* **2020**, *10*, 726.
12. Dell'Anno, M.; Hejna, M.; Sotira, S.; Caprarulo, V.; Reggi, S.; Pilu, R.; Miragoli, F.; Callegari, M.L.; Panseri, S.; Rossi, L. Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets. *Anim. Feed Sci. Tech.* **2020**, *266*, 114519, doi.org/10.1016/j.anifeedsci.2020.114519.
13. Onelli, E.; Moscatelli, A.; Gagliardi, A.; Zaninelli, M.; Bini, L.; Baldi, A.; Caccianiga, M.; Reggi, S.; Rossi, L. Retarded germination of *Nicotiana tabacum* seeds following insertion of exogenous DNA mimics the seed persistent behavior. *PLoS ONE* **2017**, *12*, e0187929, doi:10.1371/journal.pone.0187929.
14. Liu, Y.; Song, M.; Che, T.M.; Almeida, J.A.S.; Lee, J.J.; Bravo, D.; Maddox, C.W.; Pettigrew, J.E. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic *Escherichia coli*. *J. Anim. Sci.* **2013**, *91*, 5294–5306.
15. Dell'Anno, M.; Sotira, S.; Rebucci, R.; Reggi, S.; Castiglioni, B.; Rossi, L. *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts. *Ital. J. Anim. Sci.* **2020**, *19*, 103–113.
16. Reggi, S.; Giromini, C.; Dell'Anno, M.; Baldi, A.; Rebucci, R.; Rossi, L. *In vitro* digestion of chestnut and quebracho tannin extracts: Antimicrobial effect, antioxidant capacity and cytomodulatory activity in swine intestinal IPEC-J2 cells. *Animals* **2020**, *10*, 195.
17. Giromini, C.; Lovegrove, J.A.; Givens, D.I.; Rebucci, R.; Pinotti, L.; Maffioli, E.; Tedeschi, G.; Sundaram, T.S.; Baldi, A. *In vitro*-digested milk proteins: Evaluation of angiotensin-1-converting enzyme inhibitory and antioxidant activities, peptidomic profile, and mucin gene expression in HT29-MTX cells. *J. Dairy Sci.* **2019**, *102*, 10760–10771.
18. Huang, Q.; Liu, X.; Zhao, G.; Hu, T.; Wang, Y. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Anim. Nutr.* **2018**, *4*, 137–150, doi:10.1016/j.aninu.2017.09.004.
19. Cappai, M.G.; Wolf, P.; Pinna, W.; Kamphues, J. Dose-response relationship between dietary polyphenols from acorns and parotid gland hypertrophy in pigs. *Food. Nutr. Sci.* **2012**, *3*, 1261.
20. Hao, R.; Li, Q.; Zhao, J.; Li, H.; Wang, W.; Gao, J. Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets. *Livest. Sci.* **2015**, *178*, 237–242, doi:10.1016/j.livsci.2015.06.004.
21. Girard, M.; Bee, G. Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. *Animal* **2020**, *14*, 95–107.
22. Biagi, G.; Cipollini, I.; Paulicks, B.R.; Roth, F.X. Effect of tannins on growth performance and intestinal ecosystem in weaned piglets. *Arch. Anim. Nutr.* **2010**, *64*, 121–135.
23. Caprarulo, V.; Giromini, C.; Rossi, L. Review: Chestnut and quebracho tannins in pig nutrition: A review of the effects on performance and intestinal health. *Animal* **2020**.
24. Girard, M.; Hu, D.; Pradervand, N.; Neuenschwander, S.; Bee, G. Chestnut extract but not sodium salicylate decrease the severity of diarrhea and enterotoxigenic *Escherichia coli* F4 shedding in artificially infected piglets. *BioRxiv* **2019**, 15(2), 0214267.

25. Bilic-Sobot, D.; Kubale, V.; Skrlep, M.; Candek-Potokar, M.; Povse, M.; Fazarinc, G.; Skorjanc, D. Effect of hydrolysable tannins on intestinal morphology, proliferation and apoptosis in entire male pigs. *Arch. Anim. Nutr.* **2016**, *70*, 378–388, doi:10.1080/1745039X.2016.1206735.
26. Candek-Potokar, M.; Skrlep, M.; Lukac, N.; Zamaratskaia, G.; Povse, M.; Bolta, S.; Kubale, V.; Bee, G. Hydrolysable tannin fed to entire male pigs affects intestinal production, tissue deposition and hepatic clearance of skatole. *Vet. J.* **2015**, *204*, 162–167, doi:10.1016/j.tvjl.2015.02.012.
27. Brus, M.; Bavdek, S.V.; Skok, J.; Škorjanc, D. Impact of supplementing pig diet with tannins on histological characteristics of small intestine and growth performance of fattening pigs. *Acta Agric. Slov. Suppl.* **2013**, *4*, 135–138.
28. Girard, M.; Thanner, S.; Pradervand, N.; Hu, D.; Ollagnier, C.; Bee, G. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental ETEC F4 model. *PLoS ONE* **2018**, *13*, e0197878, doi:10.1371/journal.pone.0197878.
29. Tretola, M.; Maghin, F.; Silacci, P.; Ampuero, S.; Bee, G. Effect of supplementing hydrolysable tannins to a grower–finisher diet containing divergent PUFA levels on growth performance, boar taint levels in back fat and intestinal microbiota of entire males. *Animals* **2019**, *9*, 1063.
30. Decreto legislativo 26/2014 del 4 marzo 2014, n. 26. Attuazione della direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici. (14G00036) (GU Serie Generale n.61 del 14-03-2014). (Legislation decret of 26/2014 on 4 March 2014, n. 26. Implementation of the Directive 2010/63/EU on the protection of animals used for scientific purposes).
31. EU Directive 2010/6/EU, of 9 February 2010 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards mercury, free gossypol, nitrites and Mowrah, Bassia, Madhuca, 2010.32. Commission Directive 2010/6/EU of 9 February 2010 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards mercury, free gossypol, nitrites and Mowrah, Bassia, Madhuca OJ L 37, 29-32.
32. *National Research Council*, *Nutrient Requirements of Swine*; National Academies Press: **2012.** Washington D.C.
33. EU Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs. *Off. J. Eur. Union* **2008**, *316*, 36–38.
34. EU Commission Regulation 152/2009 laying down the methods of sampling and analysis for the official control of feed. *Off. J. the Eur. Union.* **2009**, - OJ L 54, p. 1-130.
35. International Organization for Standardization. *ISO-24333: Cereal and Cereal Products—Sampling*; International Organization for Standardization: Geneva, Switzerland, 2009.
36. AOAC. *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Washington, DC, USA, 2005.
37. Attard, E.A. Rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. *Open Life Sci.* **2013**, *8*, 48–53.
38. Regmi, P.R.; Ferguson, N.S.; Zijlstra, R.T. *In vitro* digestibility techniques to predict apparent total tract energy digestibility of wheat in grower pigs. *J. Anim. Sci.* **2009**, *87*, 3620–3629.
39. Castrica, M.; Rebucci, R.; Giromini, C.; Tretola, M.; Cattaneo, D.; Baldi, A. Total phenolic content and antioxidant capacity of agri-food waste and by-products. *Ital. J. Anim. Sci.* **2019**, *18*, 336–341, doi:10.1080/1828051X.2018.1529544.

40. Institute. *SAS/STAT® 9.4 User's Guide*, 2nd ed.; SAS Institute Inc.: Cary, NC, USA, 2009.
41. Klem, T.B.; Bleken, E.; Morberg, H.; Thoresen, S.I.; Framstad, T. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. *Vet. Clin. Pathol.* **2010**, *39*, 221–226.
42. Friendship, R.M.; Lumsden, J.H.; McMillan, I.; Wilson, M.R. Hematology and biochemistry reference values for Ontario swine. *Can. J. Comp. Med.* **1984**, *48*, 390.
43. IZSLER, Parametri di Chimica Clinica: Valori Osservati in Suini di Diversa Età. Available online: https://www.izsler.it/pls/izs_bs/v3_s2ew_consultazione.mostra_pagina?id_pagina=1494 (10-01-2012accessed on).
44. Mueller-Harvey, I. Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.* **2006**, *86*, 2010–2037, doi:10.1002/jsfa.2577.
45. Galassi, G.; Mason, F.; Rapetti, L.; Crovetto, G.; Spanghero, M. Digestibility and metabolic utilisation of diets containing chestnut tannins and their effects on growth and slaughter traits of heavy pigs. *Ital. J. Anim. Sci.* **2019**, *18*, 746–753, doi:10.1080/1828051X.2019.1570361.
46. Chapman, P.M. Defining hormesis: Comments on Calabrese and Baldwin. *Hum. Exp. Toxicol.* **2002**, *21*, 99–101.
47. Bee, G.; Silacci, P.; Ampuero-Kragten, S.; Candek-Potokar, M.; Wealleans, A.; Litten-Brown, J.; Salminen, J.; Mueller-Harvey, I. Hydrolysable tannin-based diet rich in gallotannins has a minimal impact on pig performance but significantly reduces salivary and bulbourethral gland size. *Animal* **2017**, *11*, 1617–1625, doi:10.1017/S1751731116002597.
48. Williams, A.; Krych, L.; Ahmad, H.; Nejsum, P.; Skovgaard, K.; Nielsen, D.; Thamsborg, S. A polyphenol-enriched diet and *Ascaris suum* infection modulate mucosal immune responses and gut microbiota composition in pigs. *PLoS ONE* **2017**, *12*, e0186546, doi:10.1371/journal.pone.0186546.
49. Carstensen, L.; Ersbøll, A.K.; Jensen, K.H.; Nielsen, J.P. *Escherichia coli* post-weaning diarrhea occurrence in piglets with monitored exposure to creep feed. *Vet. Microbiol.* **2005**, *110*, 113–123.
50. Laine, T.M.; Lyytikäinen, T.; Yliaho, M.; Anttila, M. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. *Acta Vet. Scand.* **2008**, *50*, 21.
51. Etim, N.N.; Williams, M.E.; Akpabio, U.; Offiong, E.E.A. Haematological parameters and factors affecting their values. *Agric. Sci.* **2014**, *2*, 37–47.
52. Chedea, V.; Palade, L.; Pelmus, R.; Dragomir, C.; Taranu, I. Red grape pomace rich in polyphenols diet increases the antioxidant status in key organs kidneys, liver, and spleen of piglets. *Animals* **2019**, *9*, 149, doi:10.3390/ani9040149.
53. Abarghuei, M.J.; Rouzbehan, Y.; Salem, A.Z.M.; Zamiri, M.J. Nitrogen balance, blood metabolites and milk fatty acid composition of dairy cows fed pomegranate-peel extract. *Livest. Sci.* **2014**, *164*, 72–80.
54. Chen, G.J.; Song, S.D.; Wang, B.X.; Zhang, Z.F.; Peng, Z.L.; Guo, C.H.; Zhong, J.C.; Wang, Y. Effects of forage: Concentrate ratio on growth performance, ruminal fermentation and blood metabolites in housing-feeding yaks. *Asian Austral. J. Anim.* **2015**, *28*, 1736.
55. Hăbeanu, M.; Lefter, N.A.; Gheorghe, A.; Untea, A.; Ropotă, M.; Grigore, D.-M.; Varzaru, I.; Toma, S.M. Evaluation of performance, nitrogen metabolism and tissue composition in barrows fed an n-3 PUFA-rich diet. *Animals* **2019**, *9*, 234.

56. Whang, K.; Easter, R. Blood urea nitrogen as an index of feed efficiency and lean growth potential in growing-finishing swine. *Asian- Asian Austral. J. Anim.* **2000**, *13*, 811–816, doi:10.5713/ajas.2000.811.
57. Bravo, L.; Abia, R.; Eastwood, M.A.; Saura-Calixtol, F. Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on coloic fermentation and faecal output. *Br. J. Nutr.* **1994**, *71*, 933–946.
58. Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317–333.
59. Choy, Y.; Quifer-Rada, P.; Holstege, D.; Frese, S.; Calvert, C.; Mills, D.; Lamuela-Raventos, R.; Waterhouse, A. Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins. *Food Funct.* **2014**, *5*, 2298–2308, doi:10.1039/c4fo00325j.
60. Choi, H.; Jeong, J.; Kim, D.; Lee, Y.; Kwon, H.; Kim, Y. Influence of rapeseed meal on growth performance, blood profiles, nutrient digestibility and economic benefit of growing-finishing pigs. *Asian Austral. J. Anim.* **2015**, *28*, 1345–1353, doi:10.5713/ajas.14.0802.

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Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood Metabolites and Gut Microbiota in Weaned Piglets

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Brief Introduction to the Study:

This scientific paper contributed to the scientific knowledge on innovative additives for piglets nutrition. Results of the following study paved the way for the publication of further manuscripts that evaluated the modulation of entire microbiota and the combined effect on animal health of the use of tributyrin as feed additive. The following study demonstrated the positive effect of tributyrin on animal performance, plasma metabolites and gut microorganisms' modulation in post-weaning piglets. The work provided interesting insights related to the use of tributyrin as an innovative feed additive for swine nutrition.

4.2 Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood Metabolites and Gut Microbiota in Weaned Piglets

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Simple Summary: In animal farming, alternatives to antibiotics are required due to the increase of antimicrobial resistance. In this contest, tributyrin showed the ability to

promote gut health, to modulate gut microbiota and to improve protein digestibility, leading also to higher growth performance. However, although the mode of action of tributyrin on the intestinal epithelial cells has been partially explained, its effects on lipid and protein metabolism needs to be investigated. This paper provides information about the influence of tributyrin on production traits, blood parameters, faecal microbiota and faecal protein excretion in weaned piglets.

Abstract: The aim of this study was to investigate the effects of tributyrin supplementation on the production traits, the main metabolic parameters and gut microbiota in weaned piglets. One hundred and twenty crossbred piglets (Large White × Landrace) were randomly divided into two experimental groups (six pens each; 10 piglets per pen): the control group (CTRL), that received a basal diet, and the tributyrin group (TRIB) that received the basal diet supplemented with 0.2% tributyrin. The experimental period lasted 40 days. Production traits were measured at days 14, 28 and 40. A subset composed of 48 animals (n = 4 for each pen; n = 24 per group) was considered for the evaluation of serum metabolic parameters and hair cortisol by enzyme-linked immunosorbent assay (ELISA), and faecal microbiota by real-time polymerase chain reaction (PCR). Our results showed that the treatment significantly increased body weight (BW) at day 28 and day 40 ($p = 0.0279$ and $p = 0.0006$, respectively) and average daily gain (ADG) from day 28 to day 40 ($p = 0.046$). Gain to feed ratio (G:F) was significantly higher throughout the experimental period ($p = 0.049$). Even if the serum parameters were in the physiological range, albumin, albumin/globulin (A/G) ratio, glucose and high-density lipoproteins (HDL) fraction were significantly higher in the TRIB group. On the contrary, tributyrin significantly decreased the urea blood concentration ($p = 0.0026$), which was correlated with lean gain and feed efficiency. Moreover, serum insulin concentration, which has a regulatory effect on protein and lipid metabolism, was significantly higher in the TRIB group ($p = 0.0187$). In conclusion, this study demonstrated that tributyrin can be considered as a valid feed additive for weaned piglets.

Keywords: pig nutrition; antibiotic alternatives; feed additives; butyrate; lactobacilli

1. Introduction

Even if antibiotics remain an essential tool for treating animal diseases, innovative feed additives are required in order to reduce their use in livestock due to increased antimicrobial resistance. In pig farming, the weaning phase is characterized by high levels of stress resulting in decreased feed intake, growth retardation and a

higher tendency to develop gastrointestinal diseases [1,2] that may require the use of antimicrobial compounds. Thus, alternatives include the use of feed supplements able to improve general health status by modulating the digestive process and the intestinal microbiota, such as probiotics, prebiotics and organic acids [3–5]. In light of this, short chain fatty acids (SCFA) play a fundamental role in modulating the intestinal microbial population and in promoting the digestion phase [6]. Butyrate is a SCFA that is produced by bacteria in the gut [7]. Aside from its primary function as an energy source for colonocytes, it is a strong mitosis promoter and a differentiation agent for intestinal epithelial cells [8], as it acts as a histone deacetylase (HDAC) inhibitor [9]. It showed also *in vitro* positive effects on hepatocytes [10]. Moreover, it has a strong antibacterial activity against both Gram-negative and Gram-positive pathogens [11] and therefore proves to be a valid aid for gut health maintenance. In pigs, different studies showed the improvement of growth performance, the repair of damaged intestinal tissues and the improvement of protein digestibility [12–14]. However, a decreased feed intake associated with its strong odour has been observed when high levels of butyrate were included in the diet. [15]. Tributyrin is a valid alternative to butyrate, as one molecule of tributyrin releases three molecules of butyrate directly in the small intestine, thus butyrate is rapidly adsorbed. Supplementation of tributyrin showed different *in vivo* positive effects on growth performance and gut health, also after lipopolysaccharide challenge [16–18]. The higher growth performance and the improvement of protein digestibility suggest that tributyrin could modulate protein and lipid metabolism. Thus, blood metabolites, insulin and leptin, which are positively correlated with body weight and with adipose and also muscle mass, could be modulated by tributyrin supplementation. However, no other studies investigated the effects of in-feed tributyrin on blood metabolites (such as glucose, urea and HDL), insulin, leptin and cortisol in healthy piglets. Thus, the aim of this study was to determine the effects of tributyrin supplementation on the production traits and nutrient metabolism in piglets reared in a conventional herd. In particular, the main blood metabolites, insulin, leptin, hair cortisol and protein content in faecal samples were analysed. Moreover, the effects of tributyrin on gut microbiota were evaluated.

2. Materials and Methods

2.1. The *In Vivo* Trial

The *in vivo* trial, complied with Italian regulation on animal experimentation and ethics (DL 26/2014) in accordance with European regulation (Dir. 2010/63), was approved by the animal welfare body of University of Milan (authorization number 31/2019) and performed in an intensive conventional herd, located in Lombardy

(Italy), free from diseases according to the ex A-list of the World Organization for Animal Health (Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome and salmonellosis).

One hundred and twenty crossbred piglets (Large White × Landrace) weaned at 28 ± 2 days were randomly allotted into two experimental groups, with similar conditions to those under which commercial farm animals were kept before the first day of the trial (six pens per group; 10 piglets each, 50% female and 50% male) After one week of adaptation, the control group (CTRL) received a basal diet, while the tributyrin group (TRIB) received the same basal diet supplemented with 0.2% of tributyrin (Ferraroni Mangimi SpA, Bonemerse, Italy). The iso-energetic and iso-proteic diet provided fulfilled the National Research Council NRC [19] requirements (Table 1). The experimental period lasted 40 days, considering day 0 the first day the two groups received the two different experimental diets. Piglets of both experimental groups were reared in a unique room, with environmental controlled conditions (temperature: 27 °C; humidity: 60%). In particular, the room had an unobstructed floor area available to each weaner piglet of 0.40 m^2, according to the Directive 2008/120/EC. Feed and water were provided *ad libitum*.

Table 1. Composition of the basal experimental diet. In the tributyrin (TRIB) group, 0.2% of corn was substituted with 0.2% tributyrin.

Items Basal Diet	**Basal Diet**
Ingredients	**% as Fed Basis**
Barley, meal	25.15
Wheat, meal	19.41
Corn, flakes	14.03
Corn, meal	4.85
Soybean, meal	4.65
Soy protein concentrates	4.11
Biscuits, meal	4.00
Dextrose monohydrate	3.50
Wheat middling	4.32
Whey protein concentrate	3.00
Fish, meal	2.50
Milk whey	2.50
Coconut oil	1.00
Soy oil	1.00
Plasma, meal	1.00
Organic Acids [1]	1.00
Dicalcium phosphate	0.85
Animal fats	0.70
L-Lysine	0.50
Benzoic acid	0.40
L-Threonine	0.35
DL-Methionine	0.35
Sodium Chloride	0.27

Vitamins [2]	0.25
L-Valine (96.5%)	0.15
L-Tryptophan	0.08
Flavouring [3]	0.04
Copper sulphate	0.04
Calculated Nutrient Levels [4]	
Crude protein	16.92
Ether extract	5.06
Crude fibre	3.15
Ash	5.1
DE 5 (Mc/Kg)	3.43

[1] Organic acids: formic acid, sodium formate, sorbic acid, orthophosphoric acid, calcium formate, citric acid, and fumaric acid; [2] Vitamins and vitamin-like compounds per kg: vitamin A, 10,000; vitamin D3, 1000 IU; vitamin E, 100 mg; vitamin B1, 3 mg; vitamin B2, 96.3 mg; vitamin B6, 5.8 mg; calcium D-pantothenate, 27 mg; vitamin B12, 0.040 mg; vitamin K3, 4.8 mg; biotin, 0.19 mg; niacinamide, 35 mg; folic Acid, 1.4 mg. Choline chloride 120 mg, betaine chloride 70 mg; [3] vanilla flavouring; [4] Calculation performed with Purimix software (Fabermatica, Cremona, Italy); [5] DE: digestible energy content estimated from NRC (2012).

2.2. Zootechnical Evaluation

The body weight was individually measured on day 0, day 14, day 28 and day 40. Average daily feed intake (ADFI) was calculated weekly by weighting the feed refuse of each pen (experimental unit for the zootechnical analyses). Average daily gain (ADG) was calculated from day 0 to 14, from day 14 to 28 and from day 28 to 40. Gain to feed ratio (G:F) was calculated from day 0 to 14, from day 14 to 28 and from day 28 to 40. The health status of the piglets was monitored daily and mortality was registered.

2.3. Protein Content in Faecal Samples

The Crude Protein (CP) in faecal samples, strictly depending on dietary protein level, was analysed in order to evaluate the protein excretion as indirect parameter to estimate the protein utilization and digestibility. Faecal samples were individually collected from rectal ampulla from a subset of animals (n = 48, 4 piglets for each pen, 50% female and 50% male) on day 40 and stored at -20 °C for further analyses. The samples were analysed following Official methods of analysis [20]. In particular, dry matter (DM) was obtained by inserting 2 g of faecal samples in previously weighed aluminium bags and dried in a forced-air oven at 105 °C for 24 h. The dried samples were then weighted and analysed for the protein content with the Kjeldahl method [20].

2.4. Blood Sample Collection and Biochemical Analyses

Blood was collected from the jugular vein from a subset of animals (n = 48, 4 piglets for each pen, 50% female and 50% male) randomly selected in each treatment

group on day 40. Blood samples were collected into vacuum tubes from each animal and maintained for 2 h at room temperature. All samples were centrifuged at 3000 rpm for 10 min at 4 °C. Serum was removed and the aliquots were stored at -20 °C for further analysis. The concentration of total protein (g/L), albumin (g/L), globulin (g/L), albumin/globulin (A/G ratio), urea (mmol/L), alanine aminotransferase (ALT-GPT, IU/L), aspartate aminotransferase (AST-GOT) IU/L, phosphatase alkaline (ALP) UI/L, total bilirubin (μmol/l), glucose (mmol/L), total cholesterol mmol/L, high-density lipoproteins (HDL) and low-density lipoproteins (LDL) fraction, calcium mmol/L, phosphorus (mmol/L), magnesium (mmol/L) were determined by multiparametric auto-analyser for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA).

2.5. Insulin and Leptin Evaluation by Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was collected from the jugular vein of the piglets after one hour of fasting, on day 40 during the morning and within one hour in order to have homogeneous conditions and the most representative parameters. Serum insulin and leptin were evaluated through enzyme-linked immunosorbent assay (ELISA) kits specific for pigs (CEA44Po and SEA084Po, Cloud-Clone corp, Katy, TA, USA) according to manufacturer instructions. Samples (n = 24, 2 piglets per pen, 50% female and 50% male) were diluted (1:5) for leptin determination, as suggested by the instructions, and tested as fresh weight for insulin. Absorbances were measured with a microplate reader at 450 nm (Bio-Rad 680 microplate reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and concentrations were calculated according to the respective standard curve.

2.6. Hair Cortisol Extraction and Assay

Hair samples were collected in one sampling time on the back, in the rump region, from a subset of animals (n = 48, 4 piglets per pen, 50% female and 50% male) randomly selected on day 40. According to Casal et al. [21] the hair follicle was not included in the sample, to avoid contamination from blood, and for the potential endocrine activity. The four samples were pooled in one sample that was then analyzed, for a total of 12 samples. Cortisol extraction was performed following the method of Burnett et al. [22], and partially modified according to Koren et al. [23]. At the end of extraction, the samples were centrifuged in a microcentrifuge (10 min; 3000 rpm) and 0.8 mL of the supernatants were dried using a nitrogen flow at a temperature of 45 °C and stored at -20 °C until the time of analysis.

Hair cortisol concentration was assessed using an Expanded Range High Sensitivity Salivary Cortisol ELISA kit (Salimetrics, State College, PA, USA) following previously validated protocols [21]. Concentrations were calculated using a

Labisystem Multiskan Ex (Midland, ON, Canada) microplate reader according to the relevant standard curves. Intra- and inter coefficient of variances were 8.8% and 9.3%, respectively.

2.7. DNA Extraction and Real-Time Polymerase Chain Reaction (PCR) to Determine Gut Microbiota

Bacterial DNA was extracted as previously reported by Patrone et al. [24]. Copy numbers of the 16S rRNA gene from *Escherichia coli*, *Enterobacteriaceae*, *Bifidobacterium* spp. and *Lactobacillus* spp. were quantified using previously reported primers [25–27]. Quantification was carried out in triplicate (n = 24, 2 piglets per pen, 50% female and 50% male) using the LightCycler 480 Instrument II (Roche Diagnostics, Monza, Italy). *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae* were quantified using the KAPA SYBRR FAST (Kapa Biosystems, Inc; Wilmington, MA, USA) containing a 300 nM final primer concentration. Instead, *E. coli* was quantified using the KAPA Probe FAST Master mix (Kapa Biosystems, Inc; Wilmington, MA, USA) containing 500 nM of primers and 100 nM of probe (final concentration). Primers and probe used for the quantification of *E. coli* were described by Penders et al. [25]. *Bifidobacterium infantis* ATCC 15697D and *E. coli* ATCC 700926D-5 genomic DNAs, used for preparing standard curves, were provided by the American Type Culture Collection (ATCC). Genomic DNA of *Lactobacillus fermentum* DSM20052 was obtained by extracting 5 mL of activated culture using the Genomic DNA extraction Kit (Promega) and quantified with a Qubit. Fluorometer (Invitrogen, Milan, Italy). Standard curves were obtained by 10-fold dilutions of genomic DNA for each reference genomic DNA. Results were obtained as ng of target/ng of total bacterial DNA and logarithmic transformation of real-time polymerase chain reaction (PCR) data was performed to achieve normal distribution.

2.8. Statistical Analysis

Data were analyzed using generalized linear mixed model through Proc GLIMMIX of SAS 9.4 (SAS Inst. Inc., Cary, NC, USA), and the repeated measures over time were included in the RANDOM statement. The model included the fixed effects of treatments (TRT), experimental day (D) and the interaction between the two factors was evaluated (TRT × D). The model also included the random effects of piglets nested within treatments. Tukey–Kramer studentized adjustments were used to separate treatment means within the two-way interactions. Within significant two-way interactions, slice option was used to separate means within a specific treatment and experimental days. Results are presented as least square means (LSMEANS) ± standard error (SE). Student t-test was used to analyse data obtained from the ELISA

assays and real-time PCR. Results are presented as means ± standard error (SE). Means were considered different when $p \leq 0.05$.

Results

3.1. Zootechnical Evaluation

No significant differences in the ADFI during the entire experimental period (p = 0.0946) were observed. However, the treatment had a positive effect on the BW, with significantly higher weights at day 28 and day 40 (p = 0.0279 and p = 0.0006, respectively). The ADG was significantly higher from day 28 to day 40 in TRIB group compared to the CTRL group (p = 0.046). Moreover, during the whole experimental period a significantly higher G:F in the TRIB group was noticed (p = 0.049) (Table 2). Only one piglet of the CTRL group died at day 7. By contrast, no mortality was observed in the TRIB group. No differences were observed between male and female in the parameters analyzed.

3.2. Protein Content in Faecal Samples

The average protein content in faecal samples was significantly lower in the TRIB group compared to the CTRL group (19.5 ± 1.91 and 22.8 ± 1.48 % of DM, respectively; p = 0.039, DF = 3). The data are expressed as mean ± SD.

3.3. Blood Sample Collection and Biochemical Analyses

The serum concentrations of total protein, globulin, AST-GOT, ALT-GPT, phosphatase alkaline, total bilirubin, total cholesterol, calcium, phosphorus, magnesium and the LDL fraction did not significantly differ between the two experimental groups (Table 3). Dietary addition of tributyrin significantly increased the serum concentration of albumin (p = 0.0002), A/G (p = 0.0117), glucose (p = 0.0396) and HDL fraction (p = 0.0349). Moreover, the animals fed the diet supplemented with 0.2 % tributyrin showed a significant decrease in the urea blood concentration (p = 0.0026).

Table 2. Weight gain and feed conversion rate of piglets fed on a diet supplemented with (TRIB; n = 60) or without (control group (CTRL); n = 60) 0.2 % tributyrin during the entire experimental period.

				p-Value			DF (num DF; Den DF)		
Items [1]	**CTRL**	**TRIB**	**SE**	**TRT**	**DAY**	**TRT X DAY**	**TRT**	**DAY**	**TRT X DAY**
BW (kg)				0.362	< 0.001	< 0.001	1; 10	3; 459	3; 459
day 0	8.70	8.76	1.04						
day 14	11.10	11.84	1.04						
day 28	15.40[a]	17.10[b]	1.04						
day 40	20.10[a]	23.20[b]	1.04						
ADG (kg)				0.034	< 0.001	0.125	1; 10	2; 20	2; 20
day 0–day 14	0.171	0.220	0.03						

day 14–day 28	0.309	0.375	0.03						
day 28–day 40	0.395[a]	0.509[b]	0.03						
ADFI (kg)				0.095	< 0.001	0.139	1; 10	4: 40	4; 40
day 0–day 14	0.327	0.426	0.03						
day 14–day 21	0.596	0.601	0.03						
day 21–day 28	0.797	0.901	0.03						
G:F (%)				0.049	0.189	0.160	1; 30	2; 30	2; 30
Overall	51.4[a]	56.53[b]	1.76						
day 0–day 14	52.44	50.75	3.06						
day 14–day 28	52.26	62.06	3.06						
day 28–day 40	49.54	56.79	3.06						

[1]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio; TRT, treatment; DF, degree of freedom. Data are presented as least square means (LSMEANS) ± SE. Different lowercase letters indicate significant differences between groups. Different superscripts indicate significant differences between groups (a,b: $p \leq 0.05$).

Table 3. Serum concentrations of the different parameters analysed in piglets fed on a diet supplemented with (TRIB; $n = 60$) or without (CTRL; $n = 60$) 0.2 % tributyrin.

Items [1]	CTRL	TRIB	SE	*p*-Value	DF
Total protein content (g/L)	52.87	56.82	1.78	0.1314	21
Albumin (g/L)	19.30 [a]	24.06 [b]	0.76	0.0002	21
Globulin (g/L)	33.58	32.77	1.78	0.7504	21
A/G *	0.58 [a]	0.78 [b]	0.05	0.0117	21
Urea (mmol/L)	2.18 [a]	1.08 [b]	0.23	0.0026	21
ALT-GPT * (IU/L)	38.33	32.91	1.87	0.0528	21
AST-GOT * (IU/L)	54.16	48.27	4.10	0.3218	21
ALP * (UI/L)	165.67	194.00	16.68	0.2432	21
Total bilirubin (umol/l)	1.98	1.79	0.11	0.2425	21
Glucose (mmol/L)	5.00 [a]	5.83 [b]	0.27	0.0396	21
Total cholesterol (mmol/L)	2.50	2.73	0.09	0.1008	21
Calcium (mmol/L)	2.28	2.44	0.07	0.1068	21
Phosphorus (mmol/L)	3.05	3.19	0.10	0.3253	21
Magnesium (mmol/L)	0.84	0.87	0.02	0.3726	21
HDL (mmol/L)	0.76[a]	0.88[b]	0.04	0.0349	21
LDL (mmol/L)	1.60	1.73	0.07	0.1961	21
Creatinine (μmol/L)	78.91	72.18	4.79	0.3318	21
Triglycerides (mmol/L)	0.68	0.63	0.05	0.5331	21

[1]A/G, albumin/globulin; ALT-GPT, alanine aminotransferase; AST-GOT, aspartate aminotransferase; ALP, phosphatase alkaline; DF: degree of freedom. Data are presented as least square means (LSMEANS) ± SE. Different superscripts indicate significant differences between groups ([a, b]: $p < 0.01$).

3.4. Insulin and Leptin Evaluation

Serum leptin did not differ between TRIB and CTRL groups (1215.6 ± 112.29 pg/mL and 1228.6 ± 121.09 pg/mL, respectively. $p = 0.6649$, DF = 25.8). On the contrary, insulin levels were significantly different between the treatment and the control groups ($p = 0.0187$, DF = 21.8). The average level of insulin in the TRIB group was 700.5 ± 42.35 pg/mL, while the CTRL group was 497.2 ± 67.84 pg/mL (Figure 1).

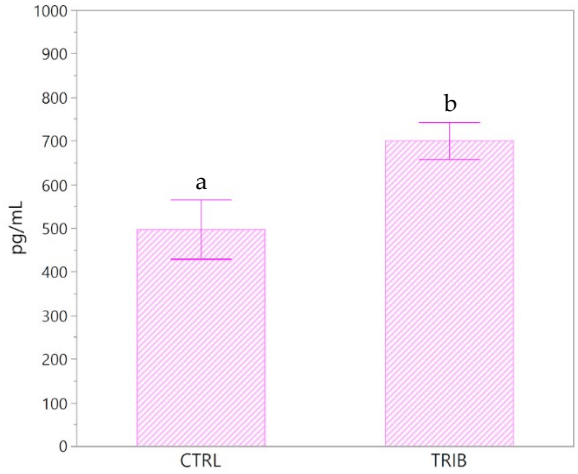

Figure 1. Serum insulin concentrations (pg/mL) in the control group (CTRL) and in the group of piglets fed a diet supplemented with 0.2 % tributyrin (TRIB). Data are presented as the mean ± SE. a,b: significant differences between groups (p = 0.0187).

3.5. Cortisol Concentration

Hair cortisol differed significantly between the TRIB and the CTRL groups (p = 0.018, DF = 10). The average level of cortisol with the standard error (SE) of the CTRL group was 9.9 ± 0.63 pg/mg, while the TRIB group had an average concentration of 13.5 ± 2.22 pg/mg (Figure 2).

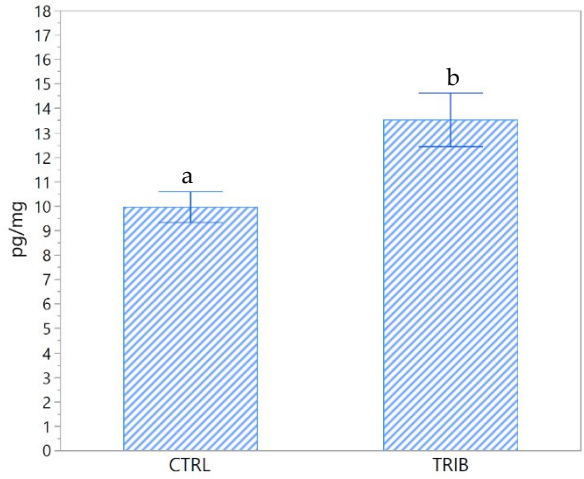

Figure 2. Hair cortisol concentrations (pg/mg) in the control group (CTRL) and in the group of piglets fed a diet supplemented with 0.2 % tributyrin (TRIB). Data are presented as the mean ± SE. a, b: significant differences between groups (p = 0.018).

3.6. DNA Extraction and Real-Time PCR to Determine Gut Microbiota

No significant differences in *E. coli* (p = 0.3823; DF = 11) and *Enterobacteriaceae* (p = 0.3217; DF = 11) content were detected between faecal samples of the two groups of animals (Figure 3). Instead, a significant reduction of lactobacilli (p = 0.0073; DF = 11) and bifidobacteria (p = 0.0003; DF = 11) was found in the TRIB group (Figure 4).

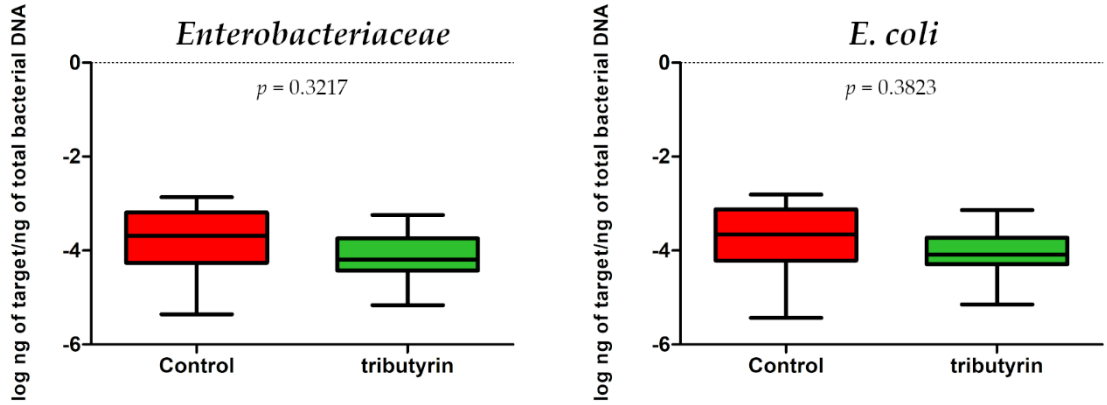

Figure 3. (a) *Enterobacteriaceae* abundance expressed as log ng of target/ng of total bacterial DNA in Table 0. % tributyrin. **(b)** *E. coli* abundance expressed as log ng of target/ng of total bacterial DNA in the faecal samples of the control group and in the group of piglets fed a diet supplemented with 0.2 % tributyrin.

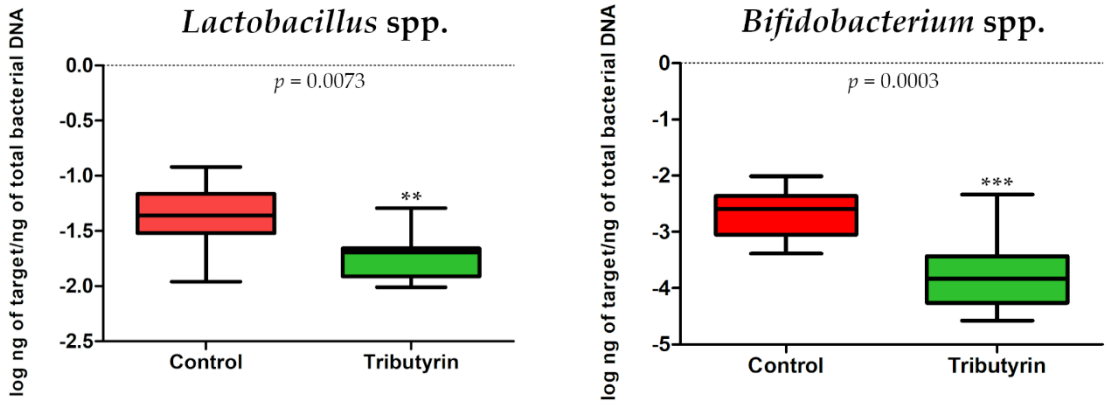

Figure 4. (a) *Lactobacillus* spp. abundance expressed as log ng of target/ng of total bacterial DNA in the faecal samples of the control group and in the group of piglets fed a diet supplemented with 0.2 % tributyrin. (**b)** *Bifidobacterium* spp. abundance expressed as log ng of target/ng of total bacterial DNA in the faecal samples of the control group and in the group of piglets fed a diet supplemented with 0.2 % tributyrin.

4. Discussion

The present study demonstrated that the tributyrin supplementation can influence positively the growth performance of healthy weaned piglets, showing the feeding effect on body weight, average daily gain, and G:F ratio. Results obtained in our study were consistent with the finding of Hou et al. [28] who found that 0.5% tributyrin in diet improved the growth rate and feed conversion ratio of piglets. Murray [29] showed that the inclusion of tributyrin at the inclusion rate of 0.25% and 0.5% increased the growth and muscle hypertrophy related to its HDAC inhibition activity. Considering our results, we can suppose that in addition to the trophic effect on muscles, the higher weights were also induced by the trophic effect of tributyrin on the gastrointestinal tract, thus resulting in a larger absorptive surface and better

growth performance. The positive effects of the dietary tributyrin are related to the release of three molecules of butyrate directly in the intestine, which is a strong mitosis promoter [8] and provides a positive effect on intestinal cell proliferation, increasing crypt depth, villi length and mucosa thickness in jejunum and ileum [30]. The increased gut health enhances also the absorption capacity, which is of greater biological value during the weaning period [14, 30]. In fact, post weaning is a critical phase in pig livestock as several stressors can compromise the health status and the productive parameters of the piglets, causing limitations in the digestive and absorptive capacity [31]. The supplementation of organic acids reduces also the gastric pH, resulting in the increase of pepsin activity, gastric retention time and improved protein digestion [32]. However, the use of butyrate instead of tributyrin led to the rapid absorption of butyrate at stomach level, which causes a reduced availability in the intestine, where it plays a pivotal role [8]. Studies in piglets showed a positive effect of dietary butyrate on growth at 0.8% inclusion levels [33], even if Lallès et al. [34] showed a decreased feed intake in piglets following increased inclusion levels of butyrate, which may be associated with its pungent odour. In this study, the dietary inclusion of 0.2% tributyrin increased the growth performance without altering the feed intake, confirming both its good tolerability and its ability of influence the feed efficiency.

Nutrition is a very important aspect of pig production which could affect blood metabolites [35]. In our study, the increase of serum glucose observed in the TRIB group compared with the CTRL group is probably associated with the higher body weights and growth performance. In fact, the glucose level is within the physiological range, according to other findings [36]. Moreover, we can suppose that butyrate, which possesses a note histone deacetylase inhibition activity [37], promoted β-cells development, proliferation and function as well as improved glucose homeostasis [38, 39]. For these reasons, it has a key role in maintaining adequate glucose level in blood [40].

The observed increase of HDL fraction occurred without affecting the total cholesterol content in blood. HDL exhibits a variety of anti-atherogenic effects, including anti-inflammatory and antioxidant activity, and the promotion of cholesterol efflux, which not only affects foam cell formation but also positively affects the reverse cholesterol transport [41,42]. Nazih et al. [43] demonstrated that butyrate was the only fatty acid that significantly increased the synthesis and the secretion of ApoA-IV protein, which is a major component of HDL and promotes the synthesis of ApoA-IV-containing HDL [44]. Xiong et al. [45] showed that tributyrin supplementation in LPS-challenged broilers increased serum HDL compared to LPS-challenged broilers without any feed supplement. Our study, together with previous results, showed that tributyrin might lead to positive effects on blood lipid regulation. ALT-GPT and AST-GOT were considered as indicator of potential liver damage. Considering our data, the tributyrin supplementation had no negative effects on hepatic functionality.

Albumin and as a consequence the albumin to globulin ratio was significantly higher in the TRIB group compared to the CTRL group. According to Dvoràk [46], albumin and A/G quotient increase gradually from birth until the post-weaning period. In particular, the better-growing piglets mostly had higher albuminemia, suggesting that nutrition, body growth and albumin synthesis are interdependent in piglets. In fact, albumin has been always linked to the nutritional status, as it rapidly increases after feeding and has always low levels during malnutrition [47]. In pigs, albumin is considered one of the most important predictors of performance, especially average daily gain and feed conversion ratio [48]. In accordance with these finding and Elbers et al. [49], our study demonstrated that piglets with higher ADFI and ADG had higher albumin levels. Moreover, in our study, insulin was significantly higher in the TRIB group compared to the CTRL group. Albumin is strongly related to insulin synthesis [50,51] and it is well known that insulin is associated with muscle protein synthesis [52,53]. Moreover, the data in our study were in the physiological range [49]. The same author demonstrated that piglets with higher serum albumin had also higher daily weight gain, in line with our study.

Urea is a waste product physiologically produced by the liver when the body breaks down proteins and high levels in blood are related to kidney or liver problems. Our results revealed that tributyrin significantly decreased the serum urea in piglets. Usually, low urea nitrogen is associated with protein deficiency in the diet [54] however the formulation of our diet fit completely the nutrient requirement of piglets. In the last decade, different studies demonstrated that plasma urea nitrogen (PUN) concentration has also a strong and inverse relationship with the lean tissue growth suggesting that the better-growing piglets have the lowest PUN values. Whang and Easter [55] also demonstrated that blood urea nitrogen (BUN) in pigs is negatively correlated with lean gain and feed efficiency. Urea production should reflect not only alterations in the dietary intake of proteins but also an animals' ability to retain dietary nitrogen in the body indicating the effective protein utilization [56]. This data, in line with the increased growth performance, is also reflected in the protein content of dried faeces and, together, these data could suggest better protein absorption and utilization. It is well known that the piglets have a relatively high gastric pH due to the scarce secretion of hydrochloric acid and pancreatic enzymes [57]. This typical condition led to limitations in the absorption and digestion processes and strategies to decrease the pH are used to overcome these problems. Organic acids can decrease the gastric pH [58] particularly tributyrin, resulting in the conversion of the pepsinogen into pepsin, thus increasing the activity of the proteolytic enzymes and improving protein digestion [16] Moreover, an *in vitro* experiment showed that butyrate enhanced the expression and activity of the peptide transporter PEPT1, located on the enterocytes, that has a key role in the protein-nitrogen absorption [59]. We suppose that in our study tributyrin ameliorated the protein absorption and utilization by the summation of different mechanisms: it principally lowered the gastric pH, thus activating the proteolytic enzymes; it increased the absorptive surface, due to its trophic effect on the

gastrointestinal tract and it probably enhanced the activity of peptide transporter on the enterocytes surface.

Insulin increases rapidly after feeding and plays a key role in regulating the assimilation of nutrients. Studies in pigs in developmental ages showed that the increase in stimulation of muscle protein synthesis in piglets is mediated by the rise of insulin in a dose-dependent way and that this response declines with adult age [52,53]. According to He et al. [60], tributyrin exerted also a regulatory effect also on lipid metabolism, as demonstrated also by our findings. In line with these literature results, our study showed that tributyrin increased the levels of insulin and this result is probably related to the higher growth performance. Moreover, several studies showed that SCFAs stimulate insulin secretion in ruminants [61] In monogastrics, butyrate increases insulin sensitivity, decreasing insulin resistance [60]. SCFAs affect pancreatic beta-cell function by directly acting as HDAC inhibitors (promoting β-cell development, proliferation, and differentiation) or indirectly, leading to insulin release [62]. However, the effects of SCFAs on insulin secretion are controversial, as some authors report that there might be no direct effect at all [63].

The increase of the cortisol concentration in stressful situations is an adaptation response of the organism to a changing environment. Studies in sheep demonstrated that ruminal infusion of a mixture of SCFAs (acetate, propionate and butyrate) increased plasma cortisol [64]. In piglets, Weber and Kerr [65] found that LPS-challenged piglets fed butyrate 0.2% had higher serum cortisol if compared to LPS-challenged piglets fed a basal diet. However, it is unknown whether butyrate infusion alone would elevate cortisol levels. Hillmann et al. [66] showed that mean cortisol levels in growing pigs were affected by the weight, and daily peaks of cortisol were higher with increasing weights. It is probable that the results of our study were related to higher body weights and not with tributyrin supplementation, however further studies are needed to better understand the role of tributyrin on cortisol level.

It is well known that dietary, environmental and social stresses induced by weaning transition are associated with microbial shifts in piglets [67]. Therefore, post-weaning is a critical period as it is associated with diarrhea and depression of growth performance. Gut microbiota plays a key role in both animal growth performance and healthy status. In this study, only *E. coli*, *Enterobacteriaceae*, *Lactobacillus* spp. and *Bifidobacterium* spp. were quantified by real-time PCR. We selected these bacterial groups because pathogens are present among the *Enterobacteriaceae* and, in particular, strains of *E. coli* that are involved in diarrhea and other diseases [68]. However, lactobacilli and bifidobacteria are beneficial bacteria in both humans and animals since the final products of their sugar fermentation play a crucial role in establishing a positive network among bacterial groups of gut microbiota [69,70]. In the pig, this consideration is partially true since *Bifidobacterium* genus represents a small portion of the total bacteria of the gut microbiota, suggesting that other microbial groups play a crucial role in conferring benefits to the healthy animal [71, 72]. Moreover, Sun et al. [73] described, in early-weaned piglets a reduction of *Lactobacillus* genus in the non-diarrheic compared to diarrheic animals. According to Sakdee et al. [16], our results

shown no significant effects of tributyrin on the populations of *E. coli* and *Enterobacteriaceae*; on the contrary a significant reduction in *Lactobacillus* spp. and bifidobacteria has been observed. Similar decreases were obtained by sodium butyrate supplementation in jejunum samples collected in chicken. Indeed, using increasing doses of the supplementation, linear significant reduction in *Lactobacillus* counts was detected and the authors suggested an inhibitory effect of butyric acid on lactobacilli growth [74]. Tributyrin is neutral but after hydrolysis, one glycerol and three butyric acid molecules are released. As butyric acid is acidic, one of the possible hypotheses is that it acts by reducing the pH of the intestinal segment in which this hydrolysis occurs. Lactobacilli are resistant to low pH, but a sensitivity strain related could justified the reduction that we detected by real time PCR. Another possible explanation has been suggested by the results of an *in vitro* study conducted by Papon et al. [75]. In this paper, tributyrin showed a strong inhibitory effect on growth of *Lactobacillus* spp. strains. Interestingly, in that same study, no effects on cell growth of one *Lactobacillus curvatus* strain were detected in the presence of glycerol and butyric acid suggesting that tributyrin itself exert inhibitory action on bacterial cells. Moreover, Gresse et al. [76] demonstrated the dynamic of piglet microbiota across the intestinal tract, with the *Lactobacillus* genus having the highest relative abundance in the stomach, duodenum and ileum segments that are also the site in which tributyrin hydrolysis occurs [16]. We can suppose that the tributyrin exerts its inhibitory effects, before the hydrolysis, on sensitive *Lactobacillus* strains inhabiting these gastrointestinal segments. Concerning bifidobacteria, no equivalent studies focused on the tributyrin sensitivity of strains belonging to this genus are available in literature, but we can suppose similar inhibitory mechanisms or maybe a combination of low pH and butyric acid concentration. It is important to underline that the reduction, although significant, does not correspond to a dramatic drop in the content of both lactobacilli and bifidobacteria. Overall, for the *Lactobacillus* genus it seems that tributyrin has highlighted its gradual reduction that normally occurs in the weaning period [77] and for this reason, we take into consideration this modification as a signal that supplementation implies a gut microbiota modification.

However, considering both the positive effect of tributyrin supplementation on animal performance and the modification of microbial populations detected, we can suppose that tributyrin influenced some different relationships between beneficial bacteria. Indeed, in weaning piglets a negative correlation between *Lactobacillus* and Residual Feed Intake (RFI) value has been described by McCormack et al. [78] indicating that this genus is correlated with a higher feed efficiency. However, no significant differences in relative abundances between high and low feed-efficient pigs were detected, indicating that other groups of bacteria play a crucial role in energy diet exploitation. On the other hand, OTU related to *Butyrivibrio*, a butyrate-producing bacterium, with high ability to ferment complex carbohydrates, was identified only in stools of high feed-efficiency piglets. These data suggest that groups of bacteria other than lactobacilli and bifidobacteria play a more relevant role in feed efficiency because of their ability to digest cereal-based diets introduced during weaning.

These microbiological aspects require further investigations in order to better understand not only the ability of tributyrin to modulate the gut microbiota composition but also the bacterial interactions that can be correlated to the growth performance and animal health.

5. Conclusions

Nowadays, a considerable number of research projects are being conducted to develop, improve and implement nonpharmaceutical approaches for enhancing animal health and performance. Our study demonstrated that tributyrin increased the growth performance and feed efficiency without affecting the feed intake, indicating a major and effective utilization of nutrients. In particular, a comprehensive look at our findings showed that tributyrin increase the protein absorption, utilization and synthesis. In fact, we found an increase of serum albumin and insulin and a decrease in serum urea and faecal protein excretion, resulting in higher ADG and G:F. This is probably due to the multiple beneficial effects of tributyrin, especially the ability to lower the gastric pH, thus activating the pepsin enzyme, and the trophic effect on the gastrointestinal tract, resulting in a larger absorptive surface, which are both of great importance in the post-weaned piglets. The higher concentration of the HDL fraction, glucose and also insulin suggested that tributyrin could have also a regulatory effect on piglets' metabolism. Despite the reduction of bifidobacteria and lactobacilli cell number, considered as beneficial groups of bacteria, animal performances and healthy status were not affected. We can conclude that tributyrin influences gut microbiota and may exhibit an inhibitory effect on some sensitive groups of bacteria. This supplementation could have beneficial effects on animal performance through the development of different relationships between groups of bacteria within the intestinal communities. All these data suggested that tributyrin could be considered a valuable feed additive for weaned piglets and, for these reasons, it will be interesting to evaluate and investigate in depth gut microbiota and bacterial interactions.

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References

1. Rossi, L.; Vagni, S.; Polidori, C.; Alborali, G.L.; Baldi, A.; Dell'Orto, V. Experimental induction of Escherichia coli diarrhoea in weaned piglets. *Open. J. Vet. Med.* **2012**, *2*, 1–8, doi:10.4236/ojvm.2012.21001.
2. Burow, E.; Rostalski, A.; Harlizius, J.; Gangl, A.; Simoneit, C.; Grobbel, M.; Käsbohrer, A. Antibiotic resistance in Escherichia coli from pigs from birth to slaughter and its association with antibiotic treatment. *Prev. Vet. Med.* **2019**, *165*, 52–62.
3. Barba-Vidal, E.; Martín-Orúe, S.M.; Castillejos, L. Practical aspects of the use of probiotics in pig production: A review. *Livest. Sci.* **2019**, *223*, 84–96.
4. Azagra-Boronat, I.; Rodríguez-Lagunas, M.J.; Castell, M.; Pérez-Cano, F.J. Prebiotics for gastrointestinal infections and acute diarrhea. *Diet. Interv. Gastrointest. Dis.* **2019**, pp. 179–191.
5. Yang, C.; Zhang, L.; Cao, G.; Feng, J.; Yue, M.; Xu, Y.; Guo, X. Effects of dietary supplementation with essential oils and organic acids on the growth performance, immune system, fecal volatile fatty acids, and microflora community in weaned piglets. *J. Anim. Sci.* **2019**, *97*, 133–143.
6. Guevarra, R.B.; Lee, J.H.; Lee, S.H.; Seok, M.J.; Kim, D.W.; Kang, B.N.; Kim, H.B. Piglet gut microbial shifts early in life: Causes and effects. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1–10.
7. Den Besten, G.; van Eunen, K.; Groen, A.K.; Venema, K.; Reijngoud, D.J.; Bakker, B.M. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* **2013**, *54*, 2325–2340.
8. Guilloteau, P.; Martin, L.; Eeckhaut, V.; Ducatelle, R.; Zabielski, R.; Van Immerseel, F. From the gut to the peripheral tissues: The multiple effects of butyrate. *Nutr. Res. Rev.* **2010**, *23*, 366–384, doi:10.1017/s0954422410000247.
9. Cuff, M.; Dyer, J.; Jones, M.; Shirazi-Beechey, S. The human colonic monocarboxylate transporter Isoform 1: Its potential importance to colonic tissue homeostasis. *Gastroenterology* **2005**, *128*, 676–686, doi:10.1053/j.gastro.2004.12.003.
10. Mátis, G.; Kulcsár, A.; Petrilla, J.; Talapka, P.; Neogrády, Z. Porcine hepatocyte-Kupffer cell co-culture as an *in vitro* model for testing the efficacy of anti-inflammatory substances. *J. Anim. Physiol. Anim. Nutr*. **2017**, *101*, 201–207.
11. Salsali, H.; Parker, W.J.; Sattar, S.A. The effect of volatile fatty acids on the inactivation of Clostridium perfringens in anaerobic digestion. *World J. Microb. Biotechnol.* **2008**, *24*, 659–665, doi:10.1007/s11274-007-9514-4.
12. Mazzoni, M.; Le Gall, M.; De Filippi, S.; Minieri, L.; Trevisi, P.; Wolinski, J.; Bosi, P. Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. *J. Nutr.* **2008**, *138*, 1426–1431, doi:10.1093/jn/138.8.142.

13. Machinsky, T.G.; Kessler, A.D.M.; Ribeiro, A.M.L.; Moraes, M.D.L.; Silva, I.C.M.D.; Cortés, M.E.M. Nutrient digestibility and Ca and P balance in pigs receiving butyric acid, phytase and different calcium levels. *Cienc. Rural* **2010**, *40*, 2350–2355.
14. Bedford, A.; Gong, J. Implications of butyrate and its derivatives for gut health and animal production. *Anim. Nutr*. **2018**, *4*, 151–159, doi:10.1016/j.aninu.2017.08.010.
15. Biagi, G.; Piva, A.; Moschini, M.; Vezzali, E.; Roth, F.X. Performance, intestinal microflora, and wall morphology of weanling pigs fed sodium butyrate. *J. Anim. Sci.* **2007**, *85*, 1184–1191.
16. Sakdee, J.; Poeikhamph, T.; Rakangthon, C.; Poungpong, K.; Bunchasak, C. Effect of tributyrin supplementation in diet on production performance and gastrointestinal tract of healthy nursery pigs. *Pak. J. Nutr.* **2016**, *15*, 954–962, doi:10.3923/pjn.2016.954.962.
17. Dong, L.; Zhong, X.; He, J.; Zhang, L.; Bai, K.; Xu, W.; Huang, X. Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets. *Clin. Nutr.* **2017**, *35*, 399–407, doi:10.1016/j.clnu.2015.03.002.
18. Song, Y.; Gu, Y.; Yin, H.; Lin, S.; Zhang, X.; Che, L.; Fang, Z. Dietary supplementation with tributyrin prevented weaned pigs from growth retardation and lethal infection via modulation of inflammatory cytokines production, ileal FGF19 expression, and intestinal acetate fermentation. *J. Anim. Sci.* **2018**, *95*, 226–238, doi:10.2527/jas.2016.0911.
19. NCR. *Nutrient Requirements of Swine*, 11th ed.; The National Academies Press: Washington, DC, USA, 2012.
20. AOAC. *Official Methods of Analysis 2005*; AOAC International: Washington, DC, USA, 2005.
21. Casal, N.; Manteca, X.; Peña, R.; Bassols, A.; Fàbrega, E. Analysis of cortisol in hair samples as an indicator of stress in pigs. *J. Vet. Behav.* **2017**, *19*, 1–6.
22. Burnett, T.A.; Madureira, A.M.L.; Silper, B.F.; Nadalin, A.; Tahmasbi, A.M.; Veira, D.M.; Cerri, R.L.A. Short communication: Factors affecting hair cortisol concentration in lactating dairy cows. *J. Dairy Sci.* **2014**, *97*, 7685–7690, doi:10.3168/jds.2014-8444.
23. Koren, L.; Mokady, O.; Karaskov, T.; Klein, J.; Koren, G.; Geffen, E. A novel method using hair for determining hormonal levels in wildlife. *Anim. Behav.* **2002**, *63*, 403–406. https://doi.org/10.1006/anbe.2001.1907.
24. Patrone, V.; Minuti, A.; Lizier, M.; Miragoli, F.; Lucchini, F.; Trevisi, E.; Rossi, F.; Callegari, M.L. Differential effects of coconut versus soy oil on gut microbiota composition and predicted metabolic function in adult mice. *BMC Genom.* **2018**, *19*, 808.
25. Penders, J.; Vink, C.; Driessen, C.; London, N.; Thijs, C.; Stobberingh, E.E. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol. Lett.* **2005**, *243*, 141–147.
26. Bartosch, S.; Fite, A.; Macfarlan, G.T.; McMurdo, M.E. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.* **2004**, *70*, 3575–3581.
27. Byun, R.; Nadkarni, M.A.; Chhour, K.L.; Martin, F.E.; Jacques, N.A.; Hunter, N. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *J. Clin. Microbiol.* **2004**, *42*, 3128–3136.
28. Hou, Y.Q.; Liu, Y.L.; Hu, J.; Shen, W.H. Effects of lactitol and tributyrin on growth performance, small intestinal morphology and enzyme activity in weaned pigs. *Asian-Australas. J. Anim*. **2006**, *19*, 1470–1477, doi:10.5713/ajas.2006.1470.

29. Murray, R.L. Tributyrin, a Butyrate Pro-Drug, as a Muscle Growth Promoter in a Porcine Model. Ph.D. Thesis, University of Maryland, College Park, MD, USA, **2018**; p. 164.
30. Kotunia, A.; Wolinski, J.; Laubitz, D.; Jurkowska, M.; Rome, V.; Guilloteau, P.; Zabielski, R. Effect of sodium butyrate on the small intestine. *J. Physiol. Pharmacol.* **2004**, *55*, 59–68.
31. Liu, Y.; Xiong, X.; Tan, B.E.; Song, M.; Ji, P.; Kim, K.; Yin, Y. Nutritional intervention for the intestinal development and health of weaned pigs. *Front. Vet.* **2019**, *6*, 46.
32. Tugnoli, B.; Giovagnoni, G.; Piva, A.; Grilli, E. From Acidifiers to Intestinal Health Enhancers: How Organic Acids Can Improve Growth Efficiency of Pigs. *Animals* **2020**, *10*, 134.
33. Piva, A.; Morlacchini, M.; Casadei, G.; Gatta, P.P.; Biagi, G.; Prandini, A. Sodium butyrate improves growth performance of weaned piglets during the first period after weaning. *Ital. J. Anim. Sci.* **2002**, *1*, 35–41, doi:10.4081/ijas.2002.35.
34. Lallès, J.P.; Bosi, P.; Janczyk, P.; Koopmans, S.J.; Torrallardona, D. Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: A review. *Animal* **2009**, *3*, 1625–1643, doi:10.1017/s175173110900398x.
35. Etim, N.N.; Offiong, E.E.; Williams, M.E.; Asuquo, L.E. Influence of nutrition on blood parameters of pigs. *Am. J. Biol. Life Sci.* **2014**, *2*, 46–52.
36. Manell, E.; Hedenqvist, P.; Svensson, A.; Jensen-Waern, M. Establishment of a refined oral glucose tolerance test in pigs, and assessment of insulin, glucagon and glucagon-like peptide-1 responses. *PLoS ONE* **2016**, *11*, e0148896, doi:10.1371/journal.pone.0148896.
37. Davie, J.R. Inhibition of histone deacetylase activity by butyrate. *J. Nutr.* **2003**, *133*, 2485S–2493S, doi:10.1093/jn/133.7.2485s.
38. Christensen, D.P.; Dahllöf, M.; Lundh, M.; Rasmussen, D.N.; Nielsen, M.D.; Billestrup, N.; Mandrup-Poulsen, T. Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus. *Mol. Med.* **2011**, *17*, 378, doi:10.2119/molmed.2011.00021.
39. Kawada, Y.; Asahara, S.I.; Sugiura, Y.; Sato, A.; Furubayashi, A.; Kawamura, M.; Koyanagi-Kimura, M. Histone deacetylase regulates insulin signaling via two pathways in pancreatic β cells. *PLoS ONE* **2017**, *12*, e0184435, doi:10.1371/journal.pone.0184435.
40. Khan, S.; Jena, G.B. Protective role of sodium butyrate, a HDAC inhibitor on beta-cell proliferation, function and glucose homeostasis through modulation of p38/ERK MAPK and apoptotic pathways: Study in juvenile diabetic rat. *Chem. Biol. Interact.* **2014**, *213*, 1–12, doi:10.1016/j.cbi.2014.02.001.
41. Podrez, E.A. Anti-oxidant properties of high-density lipoprotein and atherosclerosis. *Clin. Exp. Pharmacol. Physiol*. **2010**, *37*, 719–725, doi:10.1111/j.1440-1681.2010.05380.x.
42. Navab, M.; Reddy, S.T.; Van Lenten, B.J.; Fogelman, A.M. HDL and cardiovascular disease: Atherogenic and atheroprotective mechanisms. *Nat. Rev. Cardiol.* **2011**, *8*, 222, doi:10.1038/nrcardio.2010.222.
43. Nazih, H.; Nazih-Sanderson, F.; Krempf, M.; Michel Huvelin, J.; Mercier, S.; Marie Bard, J. Butyrate stimulates ApoA-IV-containing lipoprotein secretion in differentiated Caco-2 cells: Role in cholesterol efflux. *J. Cell. Biochem.* **2001**, *83*, 230–238, doi:10.1002/jcb.1221.
44. Duka, A.; Fotakis, P.; Georgiadou, D.; Kateifides, A.; Tzavlaki, K.; von Eckardstein, L.; Zannis, V.I. ApoA-IV promotes the biogenesis of apoA-IV-containing HDL particles with the participation of ABCA1 and LCAT. *J. Lipid Res.* **2013**, *54*, 107–115, doi:10.1194/jlr.m030114.
45. Xiong, J.; Qiu, H.; Bi, Y.; Zhou, H.L.; Guo, S.; Ding, B. Effects of Dietary Supplementation with Tributyrin and Coated Sodium Butyrate on Intestinal Morphology, Disaccharidase

Activity and Intramuscular Fat of Lipopolysaccharide-Challenged Broilers. *Braz. J. Poult. Sci.* **2018**, *20*, 707–716, doi:10.1590/1806-9061-2018-0787.
46. Dvorák, M. Changes in blood protein levels in piglets during development and during stress. *Vet. Med-Czech* **1986**, *31*, 403–414.
47. Fuhrman, M.P.; Charney, P.; Mueller, C.M. Hepatic proteins and nutrition assessment. *J. Am. Diet. Assoc*. **2004**, *104*, 1258–1264.
48. Doornenbal, H.; Tong, A.K.W.; Sather, A.P. Relationships among serum characteristics and performance and carcass traits in growing pigs. *J. Anim. Sci*. **1986**, *62*, 1675–1681.
49. Elbers, A.R.W.; Counotte, G.H.M.; Tielen, M.J.M. Haematological and clinicochemical blood profiles in slaughter pigs: Reference values, between and within herd variability and the relationship with herd factors and growth performance. *Vet. Q*. **1992**, *14*, 57–62.
50. Liang, T.J.; Grieninger, G. Direct effect of insulin on the synthesis of specific plasma proteins: Biphasic response of hepatocytes cultured in serum and hormone free medium. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6972–6976.
51. Chen, Q.; Lu, M.; Monks, B.R.; Birnbaum, M.J. Insulin is required to maintain albumin expression by inhibiting forkhead box O1 protein. *J. Biol. Chem*. **2016**, *291*, 2371–2378.
52. O'Connor, P.M.; Bush, J.A.; Suryawan, A.; Nguyen, H.V.; Davis, T.A. Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *284*, E110–E119.
53. Davis, T.A.; Suryawan, A.; Orellana, R.A.; Fiorotto, M.L.; Burrin, D.G. Amino acids and insulin are regulators of muscle protein synthesis in neonatal pigs. *Animal* **2010**, *4*, 1790–1796.
54. Figueroa, J.L.; Martínez, M.; Trujillo, J.E.; Zamora, V.; Cordero, J.L.; Sánchez-Torres, M.T. Plasma urea nitrogen concentration and growth performance of finishing pigs fed sorghum-soybean meal, low-protein diets. *J. Appl. Anim. Res*. **2008**, *33*, 7–12.
55. Whang, K.Y.; Easter, R.A. Blood urea nitrogen as an index of feed efficiency and lean growth potential in growing-finishing swine. *Asian-Australas. J. Anim.* **2000**, *13*, 811–816, doi:10.5713/ajas.2000.811.
56. Coma, J. Use of plasma urea nitrogen as a rapid response criterion to estimate the lysine requirements of growing and lactating pigs. *J. Anim. Sci*. **1995**, *73*, 472–481.
57. Suiryanrayna, M.V.; Ramana, J.V. A review of the effects of dietary organic acids fed to swine. *J. Anim. Sci. Biotechnol.* **2015**, *6*, 45.
58. Partanen, K.H.; Mroz, Z. Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* **1999**, *12*, 117–145.
59. Dalmasso, G.; Nguyen, H.T.T.; Yan, Y.; Charrier-Hisamuddin, L.; Sitaraman, S.V.; Merlin, D. Butyrate transcriptionally enhances peptide transporter PepT1 expression and activity. *PLoS ONE* **2008**, *3*, e2476.
60. He, J.; Dong, L.; Xu, W.; Bai, K.; Lu, C.; Wu, Y.; Wang, T. Dietary tributyrin supplementation attenuates insulin resistance and abnormal lipid metabolism in suckling piglets with intrauterine growth retardation. *PLoS ONE* **2015**, *10*, e0136848.
61. Husveth, F.; Szegleti, C.; Neogrady, Z. Infusion of various short chain fatty acids causes different changes in the blood glucose and insulin concentrations in growing lambs deprived of food overnight. *J. Vet. Med. Sci.* **1996**, *43*, 437–444.
62. Canfora, E.E.; Jocken, J.W.; Blaak, E.E. Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat. Rev. Endocrinol.* **2015**, *11*, 577.

63. Liu, J.L.; Segovia, I.; Yuan, X.L.; Gao, Z.H. Controversial roles of gut microbiota-derived short chain fatty acids (SCFAs) on pancreatic beta-cell growth and insulin secretion. *Int. J. Mol. Sci.* **2020**, *21*, 910.
64. Boukhliq, R.; Martin, G.B. Administration of fatty acids and gonadotrophin secretion in the mature ram. *Anim. Reprod. Sci.* **1997**, *49*, 143–159, doi:10.1016/s0378-4320(97)00065-1.
65. Weber, T.E.; Kerr, B.J. Effect of sodium butyrate on growth performance and response to lipopolysaccharide in weanling pigs. *J. Anim. Sci.* **2008**, *86*, 442–450, doi:10.2527/jas.2007-0499.
66. Hillmann, E.; Schrader, L.; Mayer, C.; Gygax, L. Effects of weight, temperature and behaviour on the circadian rhythm of salivary cortisol in growing pigs. *Animal* **2008**, *2*, 405–409, doi:10.1017/s1751731107001279.
67. Gresse, R.; Chaucheyras Durand, F.; Dunière, L.; Blanquet-Diot, S.; Forano, E. Microbiota Composition and Functional Profiling Throughout the Gastrointestinal Tract of Commercial Weaning Piglets. *Microorganisms* **2019**, *7*, 343.
68. Schierack, P.; Walk, N.; Reiter, K.; Weyrauch, K.D.; Wieler, L.H. Composition of intestinal Enterobacteriaceae populations of healthy domestic pigs. *Microbiology* **2007**, *153*, 3830–3837.
69. Yu, D.; Zhu, W.; Hang, S. Effects of long-term dietary protein restriction on intestinal morphology, digestive enzymes, gut hormones, and colonic microbiota in pigs. *Animals* **2019**, *9*, 180.
70. Li, H.; Lei, Z.; Chen, L.; Qi, Z.; Wang, W.; Qiao, J. Lactobacillus acidophilus, alleviates the inflammatory response to enterotoxigenic Escherichia coli, k88 via inhibition of the nf-κb and p38 mitogen-activated protein kinase signaling pathways in piglets. *BMC Microbiol*. **2016**, *16*, 273.
71. Wegmann, U.; MacKenzie, D.A.; Zheng, J.; Goesmann, A.; Roos, S.; Swarbreck, D.; Walter, J.; Crossman, L.C.; Juge, N. The pan-genome of Lactobacillus reuteri strains originating from the pig gastrointestinal tract. *BMC Genom.* **2015**, *6*, 1023.
72. Fouhse, J.M.; Zijlstra, R.T.; Willing, B.P. The role of gut microbiota in the health and disease of pigs. *Anim. Front*. **2016**, *6*, 30–36.
73. Heinritz, S.N.; Weiss, E.; Eklund, M.; Aumiller, T.; Louis, S.; Rings, A.; Messner, S.; Camarinha-Silva, A.; Seifert, J.; Bischoff, S.C.; et al. Intestinal microbiota and microbial metabolites are changed in a pig model fed a high-fat/low-fiber or a low-fat/high-fiber diet. *PLoS ONE* **2016**, *11*, e0154329.
74. Sun, J.; Du, L.; Li, X.; Zhong, H.; Ding, Y.; Liu, Z.; Ge, L. Identification of the core bacteria in rectums of diarrheic and non-diarrheic piglets. *Sci. Rep.* **2019**, *9*, 1–10.
75. Hu, Z.; Guo, Y. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. *Anim. Feed Sci. Technol.* **2007**, *132*, 240–249.
76. Papon, M.; Talon, R. Factors affecting growth and lipase production by meat lactobacilli strains and Brochothrix thermosphacta. *J. Appl. Bacteriol.* **1988**, *64*, 107–115.
77. Gresse, R.; Chaucheyras-Durand, F.; Fleury, M.A.; Van de Wiele, T.; Forano, E.; Blanquet-Diot, S. Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. *Trends Microbiol.* **2017**, *25*, 851–873.
78. Yang, Q.; Huang, X.; Wang, P.; Yan, Z.; Sun, W.; Zhao, S.; Gun, S. Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. *MicrobiologyOpen* **2019,** *8*, e923.

79. McCormack, U.M.; Curião, T.; Buzoianu, S.G.; Prieto, M.L.; Ryan, T.; Varley, P.; O'Sullivan, O. Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. *Appl. Environ. Microbiol.* **2017**, *83*, e00380-17.

Brief Introduction to the Study:
Based on encouraging results observed in swine species the effect of tributyrin supplementation was evaluated in pre-weaning calves. Results demonstrated the ability of 0.3% of tributyrin in-milk supplementation in the decrease of diarrhoea frequency over 42 days of trial. Observed findings were in line with previous studies confirming the bioactive activity of tributyrin for the prevention of neonatal diarrhoea in calves. These findings confirms the potential use of tributyrin as interesting alternative to antibiotics also in bovine species during the pre-weaning phase.

4.3 Evaluation of tributyrin supplementation in milk replacer on diarrhoea occurrence in pre-weaning Holstein calves

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Abstract

Neonatal calf diarrhoea (NCD) is one of the most important health challenges in cattle herds causing substantial economic losses and antimicrobial use. Due to the raising problem of antimicrobial resistance effective alternatives are urgently required, in line with European policies. The aim of this study was to evaluate the effect of tributyrin supplementation in milk replacer on diarrhoea, performance and metabolic status in pre-weaning Holstein calves. Twelve newborn calves, after colostrum administration, were randomly allotted in two experimental groups for 42 days: control (CTRL) fed milk replacer, tributyrin (TRIB) fed milk replacer supplemented with 0.3% of tributyrin on milk powder weight. Calves body weight was recorded on a weekly basis from day 7 to 42 and feed intake was recorded daily to calculate zootechnical performance. Faecal consistency was assessed daily through the faecal score (0-3 scale; considering diarrhoea moderate = 2 and severe = 3). Faecal samples were collected weekly from rectal ampulla for microbiological analysis by plate counting method evaluating the number of total bacteria, lactic acid bacteria and coliform bacteria. At day 0 and 42 individual blood sample were collected from jugular vein for metabolic profile analysis. Serum samples of day 42 were also evaluated for the antioxidant barrier using a colorimetric test, while glucagon-like peptide 2 and diamine oxidase concentrations were measured through immunoenzymatic assays. Tributyrin

supplementation did not influenced zootechnical performance of calves over 42 days of trial. Diarrhoea frequency was significantly lower in TRIB compared to CTRL group (27.91% and 38.37%; $p < 0.01$) considering the whole experimental period. In particular, TRIB group showed a significantly reduced frequency of moderate diarrhoea ($p < 0.01$) while severe diarrhoea did not differ between group, thus suggesting a preventive effect of tributyrin. Faecal total bacterial, lactic acid and coliform bacteria counts did not show differences between groups. Urea serum concentrations tended to be lower in TRIB compared to CTRL, indicating an efficient utilization of dietary protein. Antioxidant barrier and glucagon-like peptide 2 were comparable between CTRL and TRIB at day 42. Diamine oxidase concentrations were significantly decreased in TRIB compared to CTRL group after 42 days of trial ($p < 0.01$), suggesting a higher gut epithelial integrity probably due to lower diarrhoea frequency and the nourish effect of tributyrin on enterocytes. In conclusion, tributyrin could be considered as interesting functional feed additive to decrease the neonatal diarrhoea occurrence and support intestinal integrity in pre-weaning calves.

Implications

Neonatal calf diarrhoea significantly impacts the farms' profitability. Antibiotic treatments are used to limit the detrimental effects of gastrointestinal infections. The global issue of antimicrobial resistance prompted all international organizations to support their judicious use and encouraged the research for sustainable alternatives to improve animal health and decrease pathologies incidence. In this study, the effect of tributyrin supplementation in milk replacer was evaluated in pre-weaning calves. 0.3% of tributyrin supplementation significantly decreased the diarrhoea frequency and improved the intestinal integrity after 42 days. Tributyrin could be considered as interesting functional feed additive to prevent diarrhoea in calves during the pre-weaning phase.

Keywords: alternatives to antibiotics, short chain fatty acids, functional nutrition, feed additives, neonatal calf diarrhoea

Introduction

Neonatal calf diarrhoea (NCD) is a well-known and globally diffused disease in dairy farms, which negatively impacts profitability and animal welfare. NCD can be the reason of 75% of calf mortality during the pre-weaning period (Khawaskar et al., 2021). Moreover, NCD is also related to indirect losses due to higher morbidity, increased

needs and costs of treatments and growth retardation, also with negative effects on further dairy career duration and productivity (e.g. delayed first calving) (Brunauer et al., 2021). NCD is a multifactorial disease where infectious agents, such as Rotavirus, Coronavirus, *Escherichia coli*, *Salmonella* spp., *Clostridium perfrigens*, *Cryptosporidium parvum*, and animal (age, immunity, general health status) and environmental-related factors (colostral consumption, calf housing, hygienic conditions) can concur to its onset and incidence (Cho and Yoon, 2014; Windeyer et al., 2014).

To limit the incidence and detrimental effects of NCD, antibiotics are used to treat young animals (Eibl et al., 2021). Antimicrobial resistance is an important multi-faceted issue on Earth, a transversal problem for several animal species (Rossi et al., 2021), that requires to be coped with an integrated approach in line with One Health principles. Besides the ban of antibiotics as growth promoters in animal feeds (Regulation EC 1831/2003), the persistent emergence of this problem prompted the European Union to introduce more limitations for veterinary drugs use and promote their judicious use (Regulation EU 6/2019).

Thus, alternative strategies to reduce the incidence of NCD in calves are required. Beyond satisfying all the dietary requirements, nutrition can become a vehicle of functional compounds, which can sustain the calves' immune system, gastrointestinal development and functionality, thus reducing the risk of pathologies development (Grossi et al., 2021a; Grossi et al., 2021b).

Butyrate is a short-chain fatty acid, also produced by bacteria in the gut, which nourish enterocytes for their development and proliferation. In addition, butyrate exerts an antimicrobial effect against a wide range of Gram + and Gram – bacterial strains (Namkung et al., 2011; Kovanda et al., 2019). It has been demonstrated that dietary supplementation of butyrate salts can increase growth performance, stimulate gastrointestinal development, and enhance nutrient digestion in calves (Guilloteau et al., 2009; Górka et al., 2018; Liu et al., 2021). However, providing butyrate salts can be challenging due to their short half-life in blood plasma and unpleasant smell that may affect the feed palatability.

Tributyrin (butanoic acid 1,2,3-propanetriyl ester), is a flavouring feed additive composed of three butyric acid molecules, that can be used as an alternative to overcome these challenges. In this form, it has a more favourable pharmacokinetics and does not have the butyrate-distinctive odour.

Tributyrin dietary supplementation has been reported to enhance growth performance, intestinal morphology, digestive and barrier function in weaned piglets (Hou et al., 2014; Dong et al., 2016; Sotira et al., 2020). A recent study showed that tributyrin supplementation may shift gut microbiota population in weaned piglets by

increasing beta diversity and relative abundance of *Desulfovibrio* spp., *Mucipirillum* spp., *Butyrivibio* spp. and *Oscillobacter* spp. (Miragoli et al., 2021).

The effect of tributyrin supplementation in calves has been investigated by few studies with controversial findings. Tributyrin is mainly released in the gut, possibly preventing NCD and improving digestion efficiency in pre-weaned calves fed milk replacers (Moquet et al., 2016) Gorka et al., 2021). Increased animal performance has been observed with butyrate supplementation in milk replacer in pre-weaning calves (Hill et al., 2007; Guilloteau et al., 2009). On the contrary, Aurajo et al. (2013; 2015) and Inabu et al. (2019) reported a reduction of zootechnical performance by supplementing tributyrin in calves.

However, the effect of tributyrin supplementation on diarrhoea prevention has not been investigated yet. The aim of this study was to assess the effect of tributyrin supplementation in milk replacer as feed additive on diarrhoea occurrence, performance and metabolic status in pre-weaning Holstein calves.

Materials and Methods

Animal housing and experimental design

The experimental trial was approved by the Animal Welfare Organization of University of Milan (OPBA authorization n° 79/2020) and performed in accordance with European regulations.

After birth, twelve Holstein calves were fed with 4 L of high-quality colostrum by bottle feeding and housed in individual straw-bedded pens with free access to water under homogeneous environmental conditions for 42 days. After 24 hours, animals were randomly allotted in two groups, balanced per weight and sex: control group (CTRL) fed commercial milk replacer (Lattover, Veronesi Verona S.p.A., Verona, Italy) (Table 1), and tributyrin group (TRIB) fed milk replacer supplemented with 0.3% of liquid tributyrin (feed additive approved by the Reg. EC 1831/2003, code 2b09211; New Feed Team Srl, Lodi, Italy) on milk replacer powder. Dietary treatments were provided after colostrum assumption, animals fed twice per day increasing the amount of milk replacer, following routinely farm practices (Table 1). Calves fed twice per day with 10% of body weight of reconstituted milk replacer for the entire trial following animals growth curve (Amaral-Phillips et al., 2006).

Table 1. Nutritional composition of milk replacer.

Analyte	Composition (% as fed)
CP	23.00
EE	18.00
CF	0.10

Ashes	7.50
Lys	2.10
Ca	1.00
P	0.70
Na	0.50

Additives per Kg: Vitamins, pro-vitamins and substances with similar effect, Vitamin A 20000 IU, Vitamin D3 4000 IU, Vitamin E 100 mg, Vitamin C 150 mg, Vitamin B1 6 mg, Vitamin B2 12 mg, Vitamin B6 6 mg, Vitamin B12 80 mg, Niacin 30 mg, Calcium D-phantothenate 25 mg, Vitamin K3 4 mg, Betain hydrochloride 250 mg; Trace elements, Iron 75 mg, Copper 6 mg, Zinc 85 mg, Iodine 1 mg, Manganese 30 mg, Selenium 0,3 mg. DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fiber; Lys: lysine. CP: crude protein; EE: ether extract; CF: crude fiber.

Zootechnical performance, diarrhoea frequency and sample collection

Calves body weight (BW) was individually recorded weekly from day 7 to day 42. The average daily gain (ADG) was calculated by dividing weekly weight gain for the time period. Consumed milk was registered daily for the calculation of average daily feed intake (ADFI). The feed conversion ratio (FCR) was evaluated by dividing the ADG and ADFI on a weekly basis. Faecal consistency was scored on a daily basis for each animal using a four-point scale (0 = dry, 1 = normal, 2 = runny, 3 = watery) considering > 1 as diarrhoea (Santos et al., 2015; Gomez et al., 2017; Rossi et al., 2021). The frequency of diarrhoea was evaluated as the percentage of observations of animals with clinical signs of diarrhoea divided by the total observation performed over 42 days. Fecal samples were collected from rectal ampulla weekly for microbiological analysis.

At day 0 and 42 blood samples were collected from jugular vein using vacuum tubes without anticoagulants for serum metabolite profile, antioxidant barrier, and immunoenzymatic analyses.

Nutrient composition of the milk replacer

The milk replacer used in this study was analysed in triplicate in terms of principal nutrients (AOAC, 2019): dry matter (DM), ether extract (EE), crude protein (CP), ash content. Dry matter (DM) was obtained by drying milk powder in a forced air oven at 65 °C for 24 h (AOAC method 930.15). CP was determined by the Kjeldahl method (AOAC method 2001.11). EE was determined after acid hydrolysis (3N HCl) using ether extraction in the Soxtec system (AOAC method 2003.05). Ash content was obtained by incinerating samples in a muffle furnace at 550 °C (AOAC method 942.05).

Microbiological analysis of faecal samples

The faecal samples were analysed by plate counting method using three different culture media: Plate Count Agar (PCA) for total bacteria, De Man, Rogosa and Sharpe Agar (MRS) for lactic acid bacteria and Violet Red Bile Broth Agar (VRBA) for coliform bacteria. Briefly, 1 g of each sample was diluted in 9 mL of sterile 0.9% NaCl and

centrifuged (3000 rpm for 10 min) to collect the supernatant. Samples were serially diluted, and bacteria were enumerated by plate counting after 24 h of semi-anaerobic incubation at 37 °C using the overlay method for MRS and VRBA, and the inclusion method for PCA (Dell'Anno et al., 2021b). The lactobacilli:coliform ratio was calculated based on plate counting results which were expressed as $\log_{10}$ of colony-forming units per gram of dried faeces ($\log_{10}$ CFU/g).

Metabolic profile, antioxidant barrier and immunoenzymatic evaluation of blood samples

Serum samples collected at 0 and 42 days were obtained by centrifugation (3000 rpm, 15 min), and analysed using a multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA) at 37 °C. Samples were analysed for the concentration of: total protein (g/L), albumin (g/L), globulin (g/L), albumin/globulin (A/G ratio), alanine aminotransferase (ALT-GPT; IU/L), glucose (mmol/L), urea (mmol/L), creatinine (μmol/L), total bilirubin (μmol/L), total cholesterol (mmol/L), triglycerides (mmol/L), high-density lipoprotein (HDL; mmol/L), low-density lipoprotein (LDL; mmol/L), phosphorus (mmol/L), and magnesium (mmol/L).

Serum samples of day 42 were analysed through the Oxy-Adsorbent test (Diacron, Grosseto, Italy) to determine the serum antioxidant barrier according to manufacturer instruction. Endpoint absorbances were measured after 90 minutes of incubation at 37°C using a UV-Vis spectrophotometer (V630 UV-Vis, Jasco GmBH, Pfungstadt, Germany) at 546 nm.

Serum Glucagon-Like Peptide 2 (GLP2) and Diamine Oxidase (DAO) concentrations were also quantified on serum samples of 42 days by using enzyme-linked immunosorbent assay (ELISA) kits specific for bovine according to the manufacturer's instructions (Bioassay Technology Laboratory, Shanghai, China). Absorbances were measured with a microplate reader at 450 nm (Bio-Rad 680 microplate reader, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and concentrations were calculated according to the respective standard curves.

Statistical analysis

Obtained data were tested for normal distribution with the Shapiro-Wilk test using JMP Pro 15 ® (SAS Inst. Inc., Cary, NC, USA). Results of zootechnical performance and plate counting were analysed using a linear model including the fixed effect of treatment (Trt), time (Time), the interaction between treatment and time (Trt x Time), while each animal was included as random factor. Frequencies of faecal score were converted into a dichotomous variable (normal/pathological), and observed frequencies were assessed using the Chi-squared test. Serum metabolites data were

evaluated after performing analysis of covariances (ANCOVA) to adjust the initial variability of serum samples. GLP-2, DAO and Oxy-Adsorbent values of 42 days were analysed using the Student's t-test for unpaired groups. Pairwise comparisons were evaluated by using the Tukey's Honestly Significant Difference test (Tukey's HSD). Results were presented as least square means ± standard error (SE). Means or frequencies were considered statistically different when $p \leq 0.05$.

Results

Nutrient composition of the milk replacer

Milk replacer composition analysis showed results in line with product label and nutritional requirements of pre-weaning calves (Council, 2001) (Table 2).

Table 2. Analysed nutrient composition of milk replacer.

Analyte	Composition (% as fed)
DM	98.45±0.05
CP	21.16±0.72
EE	17.52±1.33
Ashes	6.61±0.07

DM: dry matter; CP: crude protein; EE: ether extract. Data are presented as means ± standard deviation.

Zootechnical performance

All calves showed no differences in zootechnical performance over the 42 days of trial (Table 3). From 7 to 42 days the BW, ADG and ADFI progressively increased without highlighting significant differences. ADG showed an average of 327.38±27.40 g/day for CTRL and 325.40±24.70 g/day for TRIB group over the 42 days of experimental trial.

Table 3. Zootechnical performance of the experimental trial (from day 7 to 42) divided by control (CTRL) and treatment (TRIB) group.

			P-values		
	CTRL	TRIB	Trt	Time	Trt x Time
BW, kg			0.8018	< 0.0001	0.9690
d 7	37.57±2.59	36.47±2.59			
d 14	38.87±2.59	38.42±2.59			
d 21	42.02±2.59	40.53±2.59			
d 28	45.03±2.59	44.18±2.59			
d 35	48.88±2.59	47.93±2.59			
d 42	52.48±2.59	51.78±2.59			
ADFI, g/day			0.6534	< 0.0001	0.9295
d 7-14	485.71±24.99	492.86±24.99			
d 14-21	529.76±24.99	557.14±24.99			
d 21-28	580.95±24.99	585.72±24.99			
d 28-35	639.29±24.99	661.90±24.99			
d 35-42	440.48±24.99	440.48±24.99			
ADG, g/day			0.4496	0.4162	0.8868
d 7-14	346.26±95.60	464.08±95.56			
d 14-21	450.00±76.84	422.77±84.83			
d 21-28	430.95±76.84	521.43±76.84			
d 28-35	550.00±76.84	535.72±76.84			
d 35-42	514.29±76.84	550.00±76.84			
FCR, g/g			0.9018	0.7193	0.4531
d 7-14	1.07±0.46	1.06±0.51			
d 14-21	1.15±0.46	0.98±0.46			
d 21-28	1.04±0.46	2.17±0.46			
d 28-35	1.50±0.46	1.11±0.46			
d 35-42	1.64±0.46	1.27±0.46			

Data are presented as least square means ± standard error of the means (SEM). BW: body weight; ADFI: average daily feed intake of milk replacer powder; FRC: feed conversion ratio; CTRL: control group fed milk replacer; TRIB: treatment group fed milk replacer supplemented with 0.3% of tributyrin (ACIFIS® Tri-B, New Feed Team, Lodi, Italy).

Diarrhoea occurrence

The diarrhoea occurrence showed a significant increase in diarrhoea frequency in CTRL group compared to TRIB group over the 42 days of experimental trial (38.37% for CTRL and 27.91% for TRIB group; $p < 0.01$). In particular, CTRL showed a higher frequency of diarrhoea during the period from 28 to 35 days compared to TRIB group ($p < 0.01$). Specifically, the frequency of moderate diarrhoea cases was significantly lower in TRIB compared to CTRL group over 42 days of trial, according to the faecal consistency score ($p < 0.01$; Figure 1). The frequency of severe diarrhoea did not differ between groups during the experimental period.

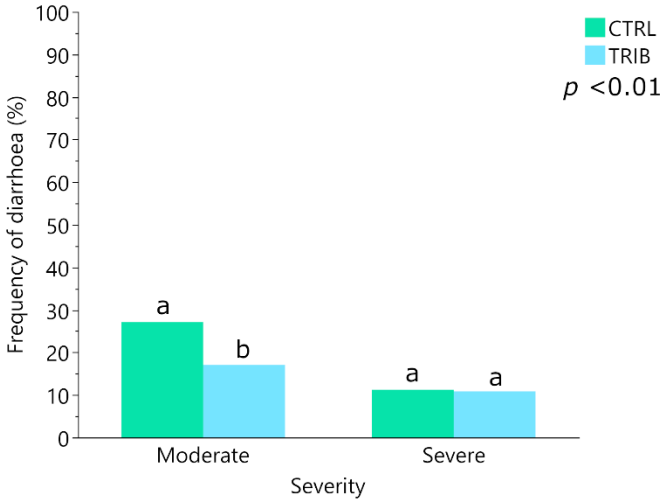

Figure 1. Frequency of moderate and severe diarrhoea cases registered on total observations during the 42 days of trial for control (CTRL) and tributyrin (TRIB) group.

Frequencies are presented as percentage of observation of animals with clinical signs of diarrhoea on total observation performed over 42 days. Moderate diarrhoea was defined as faecal score = 2 and severe diarrhoea was defined as faecal score = 3. Different lowercase letters indicate statistically significant differences between groups ($p < 0.01$).

Faecal bacteria plate counting

Plate counting of faecal samples did not show significant differences for coliform bacteria and lactic acid:coliform bacteria ratio (Table 4). Even if a significant effect of the interaction (Trt x Time) was registered for the total bacterial count and lactic acid bacteria, pairwise comparisons did not highlight any statistically significant difference. The total bacterial count of CTRL group tended to be increased at 7 days of trial compared to TRIB group (p=0.088).

Table 4. Microbiological evaluation by plate courting of faecal samples of control (CTRL) and tributyrin (TRIB) group over 42 days of trial.

				P-values		
	CTRL	**TRIB**	**SE**	Trt	Time	Trt x Time
Total bacterial count (log_{10} CFU/g of dried faeces)				0.1069	< 0.0001	0.0119
d 0	8.65	7.85	0.25			
d 7	8.63*	7.48*	0.25			
d 14	7.43	7.90	0.25			
d 21	7.12	7.38	0.25			
d 28	7.09	7.29	0.25			
d 35	7.37	6.85	0.25			
d 42	7.24	6.93	0.25			
Lactic acid bacteria (log_{10} CFU/g of dried faeces)				0.2364	< 0.0001	0.0049
d 0	8.22	7.36	0.26			
d 7	8.02	7.55	0.26			
d 14	7.28	7.61	0.26			
d 21	7.01	7.15	0.26			
d 28	6.63	7.09	0.26			
d 35	7.16	6.40	0.26			
d 42	6.18	7.07	0.26			
Coliform bacteria (log_{10} CFU/g of dried faeces)				0.8515	< 0.0001	0.4058
d 0	6.51	6.67	0.37			
d 7	7.02	6.28	0.37			
d 14	5.94	6.48	0.37			
d 21	4.84	5.60	0.37			
d 28	5.65	5.57	0.37			
d 35	5.74	5.59	0.37			
d 42	5.34	5.21	0.37			
Lactic acid:coliform bacteria ratio (log_{10} CFU/log_{10} CFU)				0.5209	0.1756	0.3687
d 0	1.29	1.14	0.09			
d 7	1.16	1.26	0.09			
d 14	1.24	1.21	0.09			
d 21	1.47	1.31	0.09			
d 28	1.18	1.27	0.09			
d 35	1.25	1.16	0.09			
d 42	1.36	1.21	0.09			

Data are presented as least square means (LSMEANS) ± standard errors (SE). *Asterisks indicate statistical tendency ($p < 0.09$).

Serum metabolic profile, antioxidant barrier level, glucagon-like peptide 2 and diamine oxidase concentrations

Serum metabolic profile displayed no significant differences between CTRL and TRIB group for all the considered parameters (Table 5). Only, the urea level in TRIB group tended to be lower compared to CTRL at 42 days of trial ($p = 0.058$).

The serum antioxidant barrier, evaluated trough Oxy-Adsorbent test, did not show significant differences between CTRL and TRIB group after 42 days (191.10±8.66 μmol HClO/mL and 211.22±8.66 μmol HClO/mL, respectively). Glucagon-like peptide 2 did not reveal statistical differences between experimental groups at day 42 of trial (97.25±3.36 ng/L for CTRL and 100.12±3.36 ng/L for TRIB group). Diamine oxidase hematic values were significantly lower in TRIB compared to CTRL group after 42 days (Figure 2; $p < 0.01$).

Table 5. Serum metabolites concentration at 42 days of trial divided by control (CTRL) and tributyrin group (TRIB).

Serum Metabolite	**CTRL**	**TRIB**	**SE**	***p-value***
Total protein content, g/L	52.55	54.55	1.48	0.3689
Albumin, g/L	30.14	30.19	0.58	0.9568
Globulin, g/L	22.75	23.98	1.27	0.5204
Albumin/Globulin, g/L	1.38	1.33	0.07	0.6493
Urea, mmol/L	2.39*	1.94*	0.14	0.0581
Non-esterified fatty acids (NEFA), mmol/L	0.26	0.35	0.03	0.1388
Glucose, mmol/L	5.14	5.11	0.37	0.9534
Total cholesterol, mmol/L	3.71	3.86	0.32	0.7497
Triglyceride, mmol/L	0.26	0.31	0.06	0.5526
Aspartate aminotransferase (AST), IU/L	52.28	52.89	2.42	0.8625
Gamma-glutamyl transferase, IU/L	38.75	25.91	10.45	0.4304
Total bilirubin, μmol/L	3.34	2.93	0.47	0.5668
Creatine kinase (CK), IU/L	173.44	139.56	24.90	0.3611
Calcium, mmol/L	2.50	2.57	0.04	0.1886
Phosphorus, mmol/L	2.48	2.52	0.08	0.7664
Magnesium, mmol/L	0.81	0.80	0.03	0.8955
Beta-hydroxybutyrate, mmol/L	0.03	0.02	0.01	0.6103
High density lipoprotein (HDL), mmol/L	1.80	1.86	0.12	0.7214
Low density lipoprotein (LDL), mmol/L	1.86	1.93	0.21	0.8235

Data are presented as least square means (LSMEANS) ± standard errors (SE). *Asterisks indicate statistical tendency ($p < 0.09$).

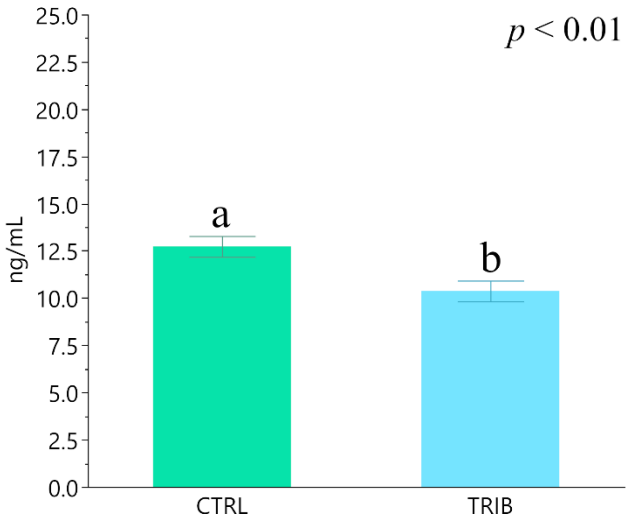

Figure 2. Diamine oxidase serum levels measured at 42 days of trial for control (CTRL) and tributyrin group (TRIB).
Data are expressed as least square means ± standard error (SE). Different lowercase letters indicate statistically significant differences between groups ($p < 0.01$).

Discussion

The present study investigated the effect of tributyrin supplementation in milk replacer on zootechnical performance, diarrhoea occurrence, metabolic, antioxidant and intestinal barrier status in pre-weaning calves.

The inclusion of tributyrin in milk replacer in pre-weaned calves did not affect the growth performance over 42 days of trial. Accordingly, Inabu et al., (2019) registered no positive effect of tributyrin supplementation in milk replacer on animal growth in pre-weaning calves. Moreover, Frieten et al. (2017) observed that butyrate sodium salts supplementation in milk replacer did not display a positive effect on ADG in pre-weaned calves. Newborn calves showed an initial decrease in BW during the first week after birth in both experimental groups. This could be ascribed to the high occurrence of diarrhoea registered from 0 to 7 days after birth (48.33% in CTRL and 36.90% in TRIB of total observations), which exert a well-known negative effect on growth performance (Karamzadeh-Dehaghani et al., 2021). After the first week, calves' growth increased progressively in both groups without showing statistical differences.

The incidence of diarrhoea was significantly lower in TRIB group over 42 days of trial. In particular, CTRL group registered an increased frequency of diarrhoea during the period from 28 to 35 days after birth. The inclusion of functional feed additives has been reported to positively impact animal health and prevent or decrease diarrhoea occurrence (Dell'Anno et al., 2021a; Stefańska et al., 2021; Abd El-Hack et al., 2022). Short-chain fatty acids are widely used as feed additives due to their antibacterial properties (Neath et al., 2022). Among them, butyrate plays a key role in the

maintenance of intestinal health and homeostasis. In particular, it has a histone deacetylase inhibitory activity that impacts gene expression by modulating chromatin structures. Several studies demonstrated that butyrate can stimulate the epigenetic regulation for anti-inflammatory and anti-carcinogenic effects (Guilloteau et al., 2010; Berni Canani et al., 2012; Du et al., 2021). In addition, butyrate can modulate the immune response by influencing the metabolism of different cell types such as macrophages (Zhou et al., 2014; Du et al., 2021). The tributyrin supplementation decreased the moderate diarrhoea occurrence over 42 days of trial, thus suggesting a modulation of intestinal homeostasis through its known effects for microbiota modulation (Miragoli et al., 2021), antimicrobial activity (Hansen et al., 2021) and trophic effect on enterocytes (Leonel et al., 2013). Tributyrin supplementation did not affect the severe diarrhoea cases, probably suggesting a lack of ability for controlling infections.

Principal faecal microbial classes revealed no difference in the present trial, suggesting that tributyrin supplementation did not alter the number of total, coliform and lactic acid bacteria. Nevertheless, Miragoli et al. (2021) showed a positive effect, by increasing the beta diversity, on gut microbiota after tributyrin supplementation for 40 days in pigs. More investigations are needed to establish the role of tributyrin supplementation on gut microbiota of pre-weaning calves.

Blood metabolic profile showed a tendency to decrease for the urea concentration in TRIB compared to CTRL group after 42 days. Urea is a final product of liver synthesis from protein metabolism, and its concentration can be considered as index of protein balance and feed efficiency (Waguespack et al., 2011). In general, an increased urea level is considered indicative of scarce nitrogen utilization, and poor protein digestibility (Pan et al., 2021; Terré et al., 2021). Besides influencing the intestinal development, butyrate supplemented through the milk replacer can also improve pancreatic functionality, with greater enzyme secretions and thus a more efficient use of feed nutrients, especially amino acids (Hill et al., 2007; Górka et al., 2018). Accordingly, Hill et al. (2007) reported a lower level of urea in pre-weaned calves supplemented with butyrate. In addition, Sotira et al. (2020) observed a decreased serum urea concentration in pigs by dietary supplementation of tributyrin for 40 days, suggesting a more efficient protein utilization.

Serum GLP-2 and Oxy-Adsorbent test data revealed no differences between experimental groups after 42 days of trial. GLP-2 is a 33 amino acids peptide produced from intestinal L cells after feed ingestion, that plays an important role in the gut morphology and functionality (Khan et al., 2017; Hatew et al., 2019) and its blood level can be a useful indicator of intestinal development (Mutanen and Pakarinen, 2017; Pyo et al., 2020). Oxy-Adsorbent test measures the blood antioxidant barrier as the capacity

of serum samples to counter the oxidative stress induced by HClO. These results suggest that tributyrin supplementation did not impair the intestinal morphology and serum antioxidant barrier, confirming also the good health status of animals at the end of the trial.

DAO concentration in blood serum was significantly lower in TRIB group after 42 days of tributyrin supplementation compared to CTRL group. DAO is an anti-histaminase enzyme produced by enterocytes and secreted into the lumen during the digestion process (Fukuda et al., 2019). Serum DAO concentration can be considered an indirect biomarker of mucosal integrity (Li et al., 2018). Indeed, higher DAO concentration in blood flow was associated with mucosal damage (Cai et al., 2019; Ren et al., 2022; Zhang et al., 2022b). Cresci et al. (2017) demonstrated the positive impact of tributyrin supplementation to reduce ethanol-induced damage supporting intestinal barrier healing from injury. Specifically, the inclusion of butyrate in milk replacer showed to modulate the gut microbiota, increase mitosis and decrease apoptotic index in the duodenum and jejunum resulting in a thicker tunica mucosa and longer intestinal villi both (Guilloteau et al., 2009; Górka et al., 2018; O'Hara et al., 2018). The observed decreased concentration of DAO could be ascribed to the positive effect of butyrate on gut epithelium integrity and the lower diarrhoea occurrence registered in TRIB group.

Conclusions

Dietary supplementation of 0.3% of tributyrin in milk replacer to Holstein calves for 42 days during the pre-weaning period decreased the diarrhoea occurrence by reducing the frequency of moderate episodes. Tributyrin tended to lower urea serum concentration, indicating an efficient protein utilization, and significantly lowered the diamine oxidase concentration in blood serum, suggesting a higher gut epithelial integrity of supplemented group compared to control after 42 days.

This study demonstrated the potential use of tributyrin as functional feed additive to prevent the neonatal calf's diarrhoea and support intestinal integrity. More studies are required to investigate the effect of tributyrin supplementation on gut microbiota in pre-weaning calves.

Ethics approval

This study was conducted following the guidelines of the Declaration of Helsinki. The experimental trial was approved by the Animal Welfare Organization of University of Milan (OPBA authorization number 79/2020).

Data and model availability statement

The data presented in this study are available within the article and from the corresponding author upon reasonable request.

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Declaration of interest

All authors declare no conflict of interests.

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References

Abd El-Hack, M.E., El-Saadony, M.T., Elbestawy, A.R., Gado, A.R., Nader, M.M., Saad, A.M., El-Tahan, A.M., Taha, A.E., Salem, H.M., El-Tarabily, K.A., 2022. Hot red pepper powder as a safe alternative to antibiotics in organic poultry feed: An updated overview. Poultry Science, 101684.

Amaral-Phillips, D.M., Scharko, P.B., Johns, J.T., Franklin, S., 2006. Feeding and managing baby calves from birth to 3 months of age. UK Cooperative Extension Service, University of Kentucky, ASC-161.

AOAC, 2019. Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC.

Araujo, G., Bach, A., Mereu, A., Ipharraguerre, I., 2013. Effects of supplementing the milk replacer with tributyrin on the metabolism of Holstein calves. XV Jornadas sobre Producción Animal, Zaragoza 14 y 15 de mayo de 2013, 300-302.

Araujo, G., Terré, M., Mereu, A., Ipharraguerre, I., Bach, A., 2015. Effects of supplementing a milk replacer with sodium butyrate or tributyrin on performance and metabolism of Holstein calves. Animal Production Science 56, 1834-1841.

Berni Canani, R., Di Costanzo, M., Leone, L., 2012. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. Clinical epigenetics 4, 1-7.

Brunauer, M., Roch, F.-F., Conrady, B., 2021. Prevalence of worldwide neonatal calf diarrhoea caused by bovine rotavirus in combination with Bovine Coronavirus, Escherichia coli K99 and Cryptosporidium spp.: a meta-analysis. Animals 11, 1014.

Cai, J., Chen, H., Weng, M., Jiang, S., Gao, J., 2019. Diagnostic and clinical significance of serum levels of D-lactate and diamine oxidase in patients with Crohn's disease. Gastroenterology Research and Practice 2019.

Cho, Y.-i., Yoon, K.-J., 2014. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. Journal of veterinary science 15, 1-17.

Council, N.R., 2001. Nutrient requirements of dairy cattle: 2001. National Academies Press.

Cresci, G.A., Glueck, B., McMullen, M.R., Xin, W., Allende, D., Nagy, L.E., 2017. Prophylactic tributyrin treatment mitigates chronic-binge ethanol-induced intestinal barrier and liver injury. Journal of gastroenterology and hepatology 32, 1587-1597.

Dell'Anno, M., Callegari, M.L., Reggi, S., Caprarulo, V., Giromini, C., Spalletta, A., Coranelli, S., Rossi, C.A.S., Rossi, L., 2021a. Lactobacillus plantarum and Lactobacillus reuteri as Functional Feed Additives to Prevent Diarrhoea in Weaned Piglets. Animals 11, 1766.

Dell'Anno, M., Reggi, S., Caprarulo, V., Hejna, M., Sgoifo Rossi, C.A., Callegari, M.L., Baldi, A., Rossi, L., 2021b. Evaluation of Tannin Extracts, Leonardite and Tributyrin Supplementation on Diarrhoea Incidence and Gut Microbiota of Weaned Piglets. Animals 11, 1693.

Dong, L., Zhong, X., He, J., Zhang, L., Bai, K., Xu, W., Wang, T., Huang, X., 2016. Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets. Clinical nutrition 35, 399-407.

Du, K., Bereswill, S., Heimesaat, M.M., 2021. A literature survey on antimicrobial and immune-modulatory effects of butyrate revealing non-antibiotic approaches to tackle bacterial infections. European Journal of Microbiology and Immunology 11, 1-9.

Eibl, C., Bexiga, R., Viora, L., Guyot, H., Félix, J., Wilms, J., Tichy, A., Hund, A., 2021. The Antibiotic Treatment of Calf Diarrhea in Four European Countries: A Survey. Antibiotics 10, 910.

Frieten, D., Gerbert, C., Koch, C., Dusel, G., Eder, K., Kanitz, E., Weitzel, J., Hammon, H., 2017. Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves. Journal of dairy science 100, 6648-6661.

Fukuda, T., Tsukano, K., Nakatsuji, H., Suzuki, K., 2019. Plasma diamine oxidase activity decline with diarrhea severity in calves indicating systemic dysfunction related to intestinal mucosal damage. Research in Veterinary Science 126, 127-130.

Grossi, S., Compiani, R., Baldi, G., Rossi, C.A.S., 2021a. The Effect of Yucca Schidigera inclusion in milk replacer for veal calves on health status, antimicrobial use and growth performance. Large Animal Review 27, 251-257.

Grossi, S., Dell'Anno, M., Rossi, L., Compiani, R., Sgoifo Rossi, C.A., 2021b. Supplementation of Live Yeast, Mannan Oligosaccharide, and Organic Selenium during the Adaptation Phase of Newly Arrived Beef Cattle: Effects on Health Status, Immune Functionality, and Growth Performance. Antibiotics 10, 1114.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., Van Immerseel, F., 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutrition research reviews 23, 366-384.

Guilloteau, P., Zabielski, R., David, J.-C., Blum, J.W., Morisset, J.A., Biernat, M., Woliński, J., Laubitz, D., Hamon, Y., 2009. Sodium-butyrate as a growth promoter in milk replacer formula for young calves. Journal of dairy science 92, 1038-1049.

Górka, P., Kowalski, Z., Zabielski, R., Guilloteau, P., 2018. Invited review: Use of butyrate to promote gastrointestinal tract development in calves. Journal of dairy science 101, 4785-4800.

Hansen, V.L., Kahl, S., Proszkowiec-Weglarz, M., Jiménez, S.C., Vaessen, S.F., Schreier, L.L., Jenkins, M.C., Russell, B., Miska, K.B., 2021. The effects of tributyrin supplementation on weight gain and intestinal gene expression in broiler chickens during Eimeria maxima-induced coccidiosis. Poultry Science 100, 100984.

Hatew, B., Inabu, Y., Sugino, T., Steele, M., 2019. Effects of pulse-dose ruminal infusion of butyrate on plasma glucagon-like peptide 1 and 2 concentrations in dairy calves. Journal of dairy science 102, 2254-2265.

Hill, T., Aldrich, J., Schlotterbeck, R., Bateman II, H., 2007. Effects of changing the fat and fatty acid composition of milk replacers fed to neonatal calves. The Professional Animal Scientist 23, 135-143.

Hou, Y., Wang, L., Yi, D., Ding, B., Chen, X., Wang, Q., Zhu, H., Liu, Y., Yin, Y., Gong, J., Wu, G., 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. British Journal of Nutrition 111, 1748-1758.

Inabu, Y., Murayama, K., Inouchi, K., Sugino, T., 2019. The effect of tributyrin supplementation to milk replacer on plasma glucagon-like peptide 2 concentrations in pre-weaning calves. Animal Science Journal 90, 1185-1192.

Karamzadeh-Dehaghani, A., Towhidi, A., Zhandi, M., Mojgani, N., Fouladi-Nashta, A., 2021. Combined effect of probiotics and specific immunoglobulin Y directed against Escherichia coli on growth performance, diarrhea incidence, and immune system in calves. Animal 15, 100124.

Khan, D., Vasu, S., Moffett, R.C., Irwin, N., Flatt, P.R., 2017. Differential expression of glucagon-like peptide-2 (GLP-2) is involved in pancreatic islet cell adaptations to stress and beta-cell survival. Peptides 95, 68-75.

Khawaskar, D.P., Sinha, D., Lalrinzuala, M.V., Athira, V., Kumar, M., Chhakchhuak, L., Mohanapriya, K., Sophia, I., Kumar, O., Chaudhuri, P., 2021. Pathotyping and antimicrobial susceptibility testing of Escherichia coli isolates from neonatal calves. Veterinary research communications, 1-10.

Kovanda, L., Zhang, W., Wei, X., Luo, J., Wu, X., Atwill, E.R., Vaessen, S., Li, X., Liu, Y., 2019. In vitro antimicrobial activities of organic acids and their derivatives on several species of gram-negative and gram-positive bacteria. Molecules 24, 3770.

Leonel, A.J., Teixeira, L.G., Oliveira, R.P., Santiago, A.F., Batista, N.V., Ferreira, T.R., Santos, R.C., Cardoso, V.N., Cara, D.C., Faria, A.M., 2013. Antioxidative and immunomodulatory effects of tributyrin supplementation on experimental colitis. British Journal of Nutrition 109, 1396-1407.

Li, F.-C., Li, Y.-K., Fan, Y.-C., Wang, K., 2018. Plasma concentration of diamine oxidase (DAO) predicts 1-month mortality of acute-on-chronic hepatitis B liver failure. Clinica Chimica Acta 484, 164-170.

Liu, W., La, A., Evans, A., Gao, S., Yu, Z., Bu, D., Ma, L., 2021. Supplementation with sodium butyrate improves growth and antioxidant function in dairy calves before weaning. Journal of Animal Science and Biotechnology 12, 1-9.

Miragoli, F., Patrone, V., Prandini, A., Sigolo, S., Dell'Anno, M., Rossi, L., Senizza, A., Morelli, L., Callegari, M.L., 2021. Implications of Tributyrin on Gut Microbiota Shifts Related to Performances of Weaning Piglets. Microorganisms 9, 584.

Moquet, P., Onrust, L., Van Immerseel, F., Ducatelle, R., Hendriks, W., Kwakkel, R., 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. World's Poultry Science Journal 72, 61-80.

Mutanen, A., Pakarinen, M.P., 2017. Serum fasting GLP-1 and GLP-2 associate with intestinal adaptation in pediatric onset intestinal failure. Clinical Nutrition 36, 1349-1354.

Namkung, H., Yu, H., Gong, J., Leeson, S., 2011. Antimicrobial activity of butyrate glycerides toward Salmonella Typhimurium and Clostridium perfringens. Poultry science 90, 2217-2222.

Neath, C., Portocarero, N., Jones, C., 2022. In vitro susceptibility of swine pathogens to feed additives and active ingredients with potential as antibiotic replacements. Journal of Applied Microbiology 132, 1713-1723.

O'Hara, E., Kelly, A., McCabe, M.S., Kenny, D.A., Guan, L.L., Waters, S.M., 2018. Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. Scientific reports 8, 1-11.

Pan, L., An, D., Zhu, W., 2021. Low-tannin sorghum grain could be used as an alternative to corn in diet for nursery pigs. Journal of Animal Physiology and Animal Nutrition 105, 890-897.

Pyo, J., Hare, K., Pletts, S., Inabu, Y., Haines, D., Sugino, T., Guan, L., Steele, M., 2020. Feeding colostrum or a 1: 1 colostrum: milk mixture for 3 days postnatal increases small intestinal development and minimally influences plasma glucagon-like peptide-2 and serum insulin-like growth factor-1 concentrations in Holstein bull calves. Journal of Dairy Science 103, 4236-4251.

Regulation EC 1831/2003, of the European Parliament and of the council, of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance).

Regulation EU 6/2019, Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC (Text with EEA relevance).

Ren, S., Chen, A., Tian, Y., Bai, Z., Wang, C., 2022. Lactobacillus paracasei from Koumiss Ameliorates Diarrhea in mice via Tight Junctions Modulation. Nutrition, 111584.

Rossi, L., Turin, L., Alborali, G.L., Demartini, E., Filipe, J.F.S., Riva, F., Riccaboni, P., Scanziani, E., Trevisi, P., Dall'Ara, P., 2021. Translational Approach to Induce and Evaluate Verocytotoxic E. coli O138 Based Disease in Piglets. Animals 11, 2415.

Sotira, S., Dell'Anno, M., Caprarulo, V., Hejna, M., Pirrone, F., Callegari, M.L., Tucci, T.V., Rossi, L., 2020. Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood Metabolites and Gut Microbiota in Weaned Piglets. Animals 10, 726.

Stefańska, B., Sroka, J., Katzer, F., Goliński, P., Nowak, W., 2021. The effect of probiotics, phytobiotics and their combination as feed additives in the diet of dairy calves on performance, rumen fermentation and blood metabolites during the preweaning period. Animal Feed Science and Technology 272, 114738.

Terré, M., Ortuzar, I., Graffelman, J., Bassols, A., Vidal, M., Bach, A., 2021. Using compositional mixed-effects models to evaluate responses to amino acid supplementation in milk replacers for calves. Journal of dairy science 104, 7808-7819.

Waguespack, A., Powell, S., Roux, M., Fruge, E., Bidner, T., Payne, R., Southern, L., 2011. Effect of determining baseline plasma urea nitrogen concentrations on subsequent posttreatment plasma urea nitrogen concentrations in 20-to 50-kilogram pigs. Journal of animal science 89, 4116-4119.

Windeyer, M., Leslie, K., Godden, S.M., Hodgins, D., Lissemore, K., LeBlanc, S., 2014. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Preventive veterinary medicine 113, 231-240.

Zhang, Z., Zhang, G., Zhang, S., Zhao, J., 2022. Fructooligosaccharide Reduces Weanling Pig Diarrhea in Conjunction with Improving Intestinal Antioxidase Activity and Tight Junction Protein Expression. Nutrients 14, 512.

Zhou, Z., Packialakshmi, B., Makkar, S., Dridi, S., Rath, N., 2014. Effect of butyrate on immune response of a chicken macrophage cell line. Veterinary Immunology and Immunopathology 162, 24-32.

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Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets

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Brief Introduction of the Study:

The following article contribution to the field of novel ingredients for sustainable animal nutrition and alternatives to antibiotics. In particular, the use of leonardite in pig nutrition is a pioneering topic with great potential due to the interesting functional characteristics of leonardite. The study highlighted that the dietary supplementation of leonardite in weaned piglets can improve growth performance and modulate the lipid metabolism. Among the most interesting results, leonardite supplementation increased serum albumin, calcium, phosphorus and magnesium concentrations suggesting a positive contribution to the protein status and a promotion of mineral uptake. In conclusion, the study proposed the inclusion of the lowest percentage of leonardite tested in literature that showed an increase of animal performance, allowing to maximise the cost-benefit of using functional compounds in feed. Leonardite represents a very interesting ingredient for improving animal growth with a positive impact on profitability and sustainability of the farm.

4.4 Evaluation of leonardite as feed additive on lipid metabolism and growth of weaned piglets

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Abstract

We evaluated the effects of leonardite supplementation, mainly composed of humic acids (HAs), as a functional feed additive in weaned piglets. One hundred and twenty piglets (Large Withe × Landrace) were weaned at 28±2 days, and randomly divided into two groups (6 pens per group, 10 piglets per pen). After one week of adaptation, for 40 days groups were fed a control diet (CTRL) and an HA enriched diet (0.25% of leonardite; HAG). Body weight (BW), average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR) were measured throughout the experimental period. On the last day of the trial four piglets per pen were randomly selected and the blood was collected to evaluate the serum metabolic profile and diamine oxidase content.

Chemical analyses showed that leonardite was characterized by a high content of ash 23.27% (as-fed basis), polyphenolic content of 35.18±3.91 mg TAEq/g, and an antioxidant capacity of 73.31±8.22 μmol TroloxEq/g.

The HAG group showed an increase in BW, ADG and ADFI ($P < 0.01$) compared to the CTRL group during the experimental period. In terms of the serum metabolic profile, the HAG group showed a significant increase in total protein content ($P < 0.001$), albumin ($P < 0.001$), albumin/globulin ratio ($P < 0.01$), phosphatase alkaline ($P < 0.01$), calcium, phosphorus and magnesium ($P < 0.05$) compared to the CTRL group.

A modulation in the serum lipid profile was recorded. The HAG group showed a decrease in total triglycerides ($P < 0.05$) with higher total cholesterol ($P < 0.05$), however only high-density lipoprotein showed a significant increase ($P < 0.001$) compared to the CTRL group. No significant differences in the amount of diamine oxidase were found between groups.

In conclusion, leonardite inclusion in the diet at 0.25% was shown to have a positive effect on the serum lipid profile and animal growth. This thus suggests that leonardite can be considered as a new feed additive, which improves the health and performance of weaned piglets.

Keywords: functional feed, humic acids, leonardite, weaned piglets, antioxidants, feed additives

1. Introduction

Weaning is recognized a stressful period in intensively reared pigs, with a high occurrence of multifactorial diseases which are the most common reason for the use of antibiotics (Zhao et al., 2007). Managing weaning correctly is crucial, as it influences

the use of antibiotics as well as long-term profitability. The European Food Safety Authority (EFSA) recommends adopting an integrated strategy in food-producing animals, reducing and also replacing the antibiotics with novel functional feed and additives (Cormican et al., 2017). Functional feed additives thus play a pivotal role, particularly concerning gastrointestinal disorders (Rossi et al., 2012; Heo et al., 2013).
Among many alternatives, leonardite which is used in veterinary practice for treating diarrhoea in horses, ruminants and poultry, has been proposed for preventing diarrhoea in animals (Ozturk et al., 2012; Domínguez-Negrete et al., 2019).
Leonardite is a microbial-derived product mainly composed of humic acids (HAs), which are derived from the decomposition of organic matter, usually exploited for the fertilization of soil. HAs also protect the mucosa of the intestine, with recognized anti-inflammatory, antiphlogistic, adsorptive and antitoxic proprieties (Islam et al., 2005; Aksu and Bozkurt, 2009).
Natural humic substances may provide benefits to piglets' health during post-weaning (Trckova et al., 2017). They have shown antioxidant proprieties that could sustain the animals during the stressful period of weaning. They have also shown antimicrobial activity against pathogens leading to a decreased incidence of diarrhoea and better growth performance also modulating the animal's metabolism (Wang et al., 2008; Aeschbacher et al., 2012).
Humic acids and their sodium salts are permitted for oral use (inclusion level: 500-2000 mg/kg of body weight) in horses, ruminants, swine and poultry for the treatment of diarrhoea, dyspepsia and acute intoxications (EGTOP/1/2011). Lower levels of humic substances used as a feed additive (2-10 g/100g of diet) in the pigs' diet seem to demonstrate that they improve growth performance and meat quality, also reducing ammonia emissions from manure (Ji et al., 2006; Wang et al., 2008; Kim et al., 2019).
Although encouraging findings concerning the use of HAs as a prophylactic tool for intestinal health have been reported, the inclusion of leonardite as a feed additive to promote growth, has not been extensively investigated. Several studies have revealed discordant findings *in vivo* probably due to the wide range of doses tested and the wide variability in the composition of humic-based products (Trckova et al., 2015; Kaevska et al., 2016; Trckova et al., 2018).
We focused on the inclusion of leonardite as a feed additive aimed at not substantially modifying the nutrient balance in the diet in order to simply be able to exploit the functional proprieties of leonartide. The aim of this study was thus to evaluate the effect of leonardite included at 0.25%, as a natural material rich in HAs, on the principal metabolic parameters and growth of weaned piglets.
Leonardite was also assessed in terms of its chemical composition, phenolic content, and antioxidant capacity.

2. Materials and Methods

2.1 Chemical evaluation of experimental diets and leonardite

The experimental diets and leonardite (Commission Regulation EU 2017/1017), registration number 13.10.2; purchased from New Feed Team S.R.L. (Lodi, Italy) were characterized in terms of their principal constituents: humidity, ether extract (EE), crude protein (CP), crude fiber (CF), and ash contents. The samples were analysed in duplicate following official analysis methods (AOAC, 2005). Dry matter (DM) was obtained by inserting samples into previously weighed aluminium bags, which were dried in a forced-air oven at 105°C for 24 h (AOAC method 930.15).

Ash was obtained using a muffle furnace at 550°C (AOAC method 942.05). CP was determined by a Kjeldahl method (AOAC method 2001.11). EE was determined using ether extraction in the Soxtec system (DM 21/12/1998). CF was determined by the filtering bag technique (AOCS method Ba 6a-05).

The mineral composition of the two experimental diets and leonardite were evaluated after mineralization with inductively coupled plasma mass spectrometry (ICP-MS). First, calibration curves for each element considered (Na, Mg, Al, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Pb, P) were obtained using certified reference materials. Dried samples (0.3 g) were digested by a microwave digestion system (Anton Paar MULTIWAVE-ECO) in Teflon tubes filled with 10 mL of 65% HNO_3 by applying a one-step temperature ramp (at 120°C in 10 min and maintained for 10 min).

The mineralized samples were cooled for 20 minutes and then transferred into the polypropylene test tubes. Mineralized samples were diluted 1:100 with 0.3 M HNO_3 in MilliQ Water. The concentration of elements was measured by ICP-MS (BRUKER Aurora-M90 ICP-MS). In order to check the nebulization performance, an aliquot of a 2 mg/L of an internal standard solution (^{72}Ge, ^{89}Y, ^{159}Tb) was added to the samples to produce a final concentration of 20 μg/L. Polyatomic interference was removed using a collision-reaction interface (CRI) with an H_2 flow of 80 mL/min through skimmer cone. The fatty acid profile of the experimental diets was analysed starting with the total lipid extraction and preparation of the fatty acid methyl esters (Christie and Han, 2003). The fatty acid analysis was carried out using gas chromatography (TRACE GC Ultra, Thermo Fisher Scientific, Rodano, Italy) fitted with an automatic sampler (AI 1300, Thermo Fisher Scientific) and flame ionization detector (FID). An RT-2560 fused silica capillary column (100 m × 0.25 mm × 0.25 μm film thickness; Restek, Milan, Italy) was used with a programmed temperature from 80°C to 180 °C at 3°C/min, then from 180 °C to 250°C at 2.5 °C/min, which was then held for 10 min. The carrier gas was helium at 1.0 mL/min with an inlet pressure of 16.9 psi. A quantitative procedure was used where 1 mL of internal standard (1 mg/mL 23:0 methyl ester; N-23-M; Nu-Chek

Prep Inc., Elysian, MN, USA) was added prior to methylation. The fatty acid methyl ester (FAME) contents were quantified by weight as a percentage of the total FAMEs. All analyses were performed in duplicate.

2.2. Polyphenolic content of leonardite

The phenolic content of leonardite was evaluated by the Folin-Ciocalteu method following Attard et al. (2013). Polyphenolic extract was obtained by diluting 2.5 g of sample with 15 mL of methanol (100%), which was then allowed to macerate for 24 h at room temperature. The samples were centrifugated (3000 rpm, 10 min) and the supernatants were collected and stored at -20° C for further polyphenol evaluation. The assay was performed by reacting 50 μL of extracted sample/standard with 500 μL of Folin-Ciocalteu reagent (10% in water) and 400 μL sodium carbonate (1 M). The reaction mixture was left to stand for 15 min in the dark and the total phenolic content was determined spectrophotometrically at 630 nm (JASCO V-630 UV-VIS, Germany). Calibration curves were prepared with tannic acid from 480 μg/mL to 15 μg/mL as standard. The results were expressed as mg/100g of tannic acid equivalent (mg TAEq/100g).

2.3. Trolox Equivalent Antioxidant Capacity assay (TEAC)

Leonardite was evaluated for its antioxidant proprieties using an ABTS radical cation discoloration assay (Prior et al., 2005). First the dried samples were diluted in water (100 mg/mL) and adjusted from an initial pH of 3.5 to pH 9 by adding NaOH (1M) thus facilitating the solubilization of humic acids. The sample was then stirred for 24 h at room temperature. The solution was centrifuged for 10 min at 3000 rpm, the supernatant was collected and stored at -20°C until the analysis. Before proceeding with the assay, the humic acid extract was adjusted to pH 7 with HCl (1M), filtered with an 0.45 μm syringe filter, and diluted in order to obtain a clear solution that would not alter the reading of the spectrophotometer. In order to assess a possible dose related antioxidant effect, the concentrations tested were: 5%, 2%, 1.25% of the humic acid original extract (Dell'Anno et al., 2020). The antioxidant activity was tested by adopting the ABTS assay, according to Prior et al. (2005).

The reaction mixture with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was generated by the reaction of 7 mM ABTS with 2.45 mM of K-persulfate. The reaction mixture was left to stand in the dark for 16 hours at room temperature and used within two days. The working solution of the $ABTS^{\bullet+}$ radical cation was obtained by diluting $ABTS^{\bullet+}$ in ethanol in order to obtain an absorbance of 0.700 ± 0.02 OD at 734 nm at room temperature. First, a calibration curve was obtained using different concentrations (2000 μM, 1500 μM, 1000 μM, 500 μM, 100 μM, 0 μM) of

Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as standard. The assay was performed using 10 μL of diluted sample (standard and extract) added to 1 mL of working solution ($ABTS^{\bullet+}$). The absorbances were recorded spectrophotometrically (JASCO V-630 UV-VIS, Germany) at 734 nm from 1 to 6 minutes. The total antioxidant capacity was expressed as the percentage inhibition (PI), according to the equation: $PI = [(AbsABTS^{\bullet+} - Abs\ sample) / AbsABTS^{\bullet+}] \times 100$, where $AbsABTS^{\bullet+}$ denotes the initial absorbance of diluted $ABTS^{\bullet+}$, and the Abs sample denotes the absorbance of the sample at every 6 mins of reaction. Using appropriate calibration curves, the result was expressed as the equivalent concentration of μmol Trolox/g after six minutes of reaction (μmol TroloxEq/g).

2.4. Experimental design and sample collection

The experimental trial was approved by the Animal Welfare Body of the University of Milan (protocol No. 31/2019) and performed in accordance with European regulations (Commission Directive 2010/6/EU). It was conducted on a commercial farm that was free from pathologies included in the ex-list A of the World Organization for Animal Health (Porcine Reproductive Respiratory Syndrome, atrophic rhinitis, transmissible gastroenteritis, salmonellosis, Aujeszky disease). A total of 120 crossbred piglets (Large White × Landrace), weaned at 28±2 days (50% female and 50% male), were housed in 12 different pens, in homogeneous environmental conditions (27°C and 60% relative humidity). In order to guarantee a homogeneous weight intra-pen and inter-group, the piglets (10 animals/pen) were randomly assigned to the control group (CTRL: 6 pens, 60 piglets) and treatment with an HA enriched diet (0.25% of leonardite; HAG: 6 pens, 60 piglets). The two experimental isoproteic and isoenergetic diets (Table 1) were formulated using Plurimix software (Fabermatica, Cremona, Italy) in order to meet the nutritional requirements for post-weaned piglets (NRC, 2012) and were provided by Ferraroni S.p.a. (Cremona, Italy). Both experimental diets were formulated including 1% coconut oil (Table 1), a raw ingredient characterized by high digestibility, as an enhancer of palatability for young animals, taking into account the balance in the saturated and unsaturated fatty acid ratio. After an adaptation period of seven days with the same basal diet, piglets were fed the experimental diets (CTRL and HAG) *ad libitum* from day 0 to day 40. Body weight (BW) was individually recorded using a validated scale at the beginning (d 0), day 14 (d 14), day 28 (d 28) and day 40 (d 40). Feed intake was recorded weekly for each pen by measuring the feed refuse per pen. Based on the BW results, the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated. In terms of zootechnical performance, each pen was considered as the experimental unit. At day 40, four animals from each pen were randomly selected, in order to collect faeces from the rectal ampulla of four animals per pen (total samples CTRL: n=24; total samples:

HAG n=24), and blood samples from the jugular vein of four animals per pen (total samples CTRL: n=24; total samples: HAG n=24) using vacuum tubes. The health status was evaluated daily and individual faecal consistency was scored weekly using a four-level scale: 0 = normal (faeces firm and well formed), 1 = soft consistency (faeces soft and formed), 2 = mild diarrhoea (loose faeces, usually yellowish), 3 = severe diarrhoea (faeces watery and projectile). A faecal consistency score ≤ 1 (0, 1) was considered normal, whereas a faecal score > 1 (2, 3) was defined as indicative of diarrhoea (Rossi et al., 2014).

Table 1. Diet composition of *in vivo* trial (% as fed basis) divided by control (CTRL) and treatment group fed with HA enriched diet (0.25% leonardite).

Items	**CTRL**	**HAG**
Ingredients, % as fed basis		
Barley, meal	25.15	25.15
Wheat, meal	19.41	19.36
Corn, flakes	14.03	13.83
Corn, meal	4.85	4.85
Soybean, meal	4.65	4.65
Soy protein concentrates	4.11	4.11
Biscuits, meal	4.00	4.00
Dextrose monohydrate	3.50	3.50
Wheat middling	4.32	4.32
Whey protein concentrate	3.00	3.00
Fish, meal	2.50	2.50
Milk whey	2.50	2.50
Coconut oil	1.00	1.00
Soy oil	1.00	1.00
Plasma, meal	1.00	1.00
Organic Acids[1]	1.00	1.00
Dicalcium phosphate	0.85	0.85
Animal fats	0.70	0.70
L-Lysine	0.50	0.50
Benzoic acid	0.40	0.40
L-Threonine	0.35	0.35
DL-Methionine	0.35	0.35
Sodium Chloride	0.27	0.27
Vitamins[2]	0.25	0.25
L-Valine (96.5%)	0.15	0.15
L-Tryptophan	0.08	0.08
Flavouring[3]	0.04	0.04
Copper sulphate	0.04	0.04
Leonardite	-	0.25
Calculated nutrient levels[4], % as fed basis		
Crude protein	16.92	16.88
Ether extract	5.06	5.19
Crude fiber	3.15	3.22
Ashes	5.1	5.1
DE[5] (Mc/Kg)	3.43	3.43

[1]Organic Acids: formic acid, sodium formate, sorbic acid, orthophosphoric acid, calcium formate, citric acid, and fumaric acid.
[2]Vitamins and vitamin-like compounds per kg: Vitamin A, 10,000; Vitamin D3, 1,000 IU; Vitamin E, 100 mg; Vitamin B1, 3 mg; Vitamin B2, 96.3 mg; Vitamin B6, 5.8 mg; Calcium D-pantothenate, 27 mg; Vitamin B12, 0.040 mg; Vitamin K3, 4.8 mg; Biotin, 0.19 mg; Niacinamide, 35 mg; Folic Acid, 1.4 mg. Choline chloride 120 mg, Betaine chloride 70 mg.
[3]Vanilla flavouring.
[4]Calculation performed with Purimix software (Fabermatica, Cremona, Italy).
[5]DE: digestible energy content estimated from NRC (2012).
HA: humic acids; HAG: humic acid enriched diet group supplemented with 0.25% leonardite.

2.5. Biological sample analysis

Blood samples (4 animals/pen) were allowed to clot for 2 hours at room temperature. Serum samples were obtained by centrifugation and were evaluated by a multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA) at 37 °C for the following metabolic parameters: total protein content, albumin, globulin, albumin/globulin (A/G), urea, alanine aminotransferase (ALT-GPT), aspartate aminotransferase (AST-GOT), phosphatase alkaline (ALP), total bilirubin, glucose, total cholesterol, calcium (Ca), phosphorus (P), magnesium (Mg), total triglycerides, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and creatinine.

Diamine oxidase concentrations in serum samples (dilution 1:10), as an indirect marker of intestinal integrity (Zhang et al., 2013), were evaluated using a DAO enzyme-linked immunosorbent assay (DAO; Wuhan Fine Biotech Co., Ltd., China). The following conditions were used: 96-well pre-coated microplates were washed twice, after which 100 μL of standard or sample were added to each well and incubated at 37°C for 90 min. The plates were washed and 100 μL of diluted detection antibody were added to each well and incubated at 37°C for 60 min. After washing, 90 μL of TMB substrate solution were added to each well. The plate was left in the dark at 37°C for 15 min, then the reaction was stopped by adding 50 μL of stop solution to each well. Absorbance was measured on a plate reader at 450 nm (Bio-Rad 680 Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, California, USA). For each plate, a calibration curve was adopted to calculate the DAO concentration of each sample using Curve Expert v. 1.4 software. The concentrations determined were expressed as nanograms of DAO per mL (ng/mL).

Collected fecal samples were analysed by RT-PCR to estimate the bacterial DNA abundance of the main enteric microorganisms as the parameter of gut health. Bacterial DNA was extracted as reported by Patrone et al. (2018). Copy numbers of the 16S rRNA gene from *Escherichia coli*, Enterobacteriaceae, *Bifidobacterium* spp. and *Lactobacillus* spp. were quantified using previously reported primers (Penders et al.

2005, Bartosch et al. 2004, Byun et al., 2004). Quantification was carried out in triplicate using the LightCycler 480 Instrument II (Roche Diagnostics, Monza, Italy). *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae were quantified using the KAPA SYBRR FAST (Kapa Biosystems, Inc; Wilmington, MA) containing a 300 nM final primer concentration. On the other hand, *E. coli* was quantified using the KAPA Probe FAST Master mix (Kapa Biosystems, Inc; Wilmington, MA) containing 500nM of primers and 100 nM of the probe (final concentration). The primers and probes used for the quantification of *E. coli* were described by Penders et al (2005). *Bifidobacterium infantis* ATCC 15697D and *E. coli* ATCC 700926D-5 genomic DNAs, used for preparing standard curves, were provided by the American Type Culture Collection (ATCC). Genomic DNA of *Lactobacillus fermentum* DSM20052 was obtained by extracting 5 mL of activated culture using the Genomic DNA extraction Kit (Promega) and quantified with a Qubit™ fluorometer (Invitrogen, Milan, Italy). Standard curves were obtained by 10-fold dilutions of genomic DNA for each reference genomic DNA. Results were expressed as ng of target DNA/ng of total fecal bacterial DNA. The four samples analysed for each pen were considered as replicates for each treatment.

2.6. Statistical analysis

Data on zootechnical performance, biological samples, antioxidant capacity and diamine oxidase were evaluated using SAS 9.4 (SAS Inst. Inc., Cary, NC). Before analyses, all data were tested for normality with the Shapiro-Wilk test (for values > 0.9 data were considered normally distributed).

The data were analysed with the general linear model. The model included the effect of treatments, and for the zootechnical performance, the effect of time (Day) and the interaction between treatment and time (TRT x Day) were included. Tukey-Kramer studentized adjustments were used to separate the means, and the results were reported as LSMEANS and SEM or standard error (SE). Means were considered different when $P \leq 0.05$ and tended to different if $0.05 < P \leq 0.1$.

3. Results and Discussion

3.1. Chemical characterization of experimental diets and leonardite

Leonardite is a heterogeneous mixture of polydisperse material formed by humification. The variability of the environment during humification significantly influences the final composition of leonardite. This is confirmed by the wide heterogeneity of commercial products for animal nutrition. The chemical characterization of leonardite enabled our results to be compared with other findings.

Our results revealed that the main component of leonardite was ash, corresponding to 23.27%, confirming the high content of minerals although a wide range from 15 to 70% on dry basis is reported in the literature (Chammui et al., 2014). Our experimental diets showed a mineral content in line with regulations (Regulation EC 1831/2003). Nevertheless, the high level of minerals did not notably alter the mineral levels as a result of the inclusion of 0.25% of leonardite in the diet, considering the maximum levels permitted by Reg. EU 1831/2011. This percentage inclusion also reduced the dustiness and obtained optimal mixing conditions during the feed production phases. The inclusion of leonardite did not affect the fatty acid profile of the diets (Table 2).
Leonardite showed a high content of Ca, Fe and Al. The contaminants (As, Pb, Cd) were scarce and below the safety limits (Commission Regulation EU 1275/2013). Moreover, no contaminants (under the detection limits) were revealed in the diet supplemented with 0.25% leonardite.
In order to reduce the emission of heavy metals into the environment and optimize the diet for a better exploitation of macro and microelements, the level of minerals in feed ingredients should always be evaluated in order to guarantee the correct diet formulation (Hejna et al., 2019) (Table 3).

3.2. Polyphenolic content of leonardite

Results from the Folin-Ciocalteu method revealed that leonardite was characterized by 35.18±3.91 mg TAEq/100g of polyphenols. Polyphenols such as hydroxytyrosol from olive oil, isoflavones from soy proanthocyanidins in grape seed extracts (Mennen et al., 2005), were evaluated as indirect indicators of the antioxidant proprieties of humic substances. The recommended dose of polyphenol intake in the human diet is 396 mg/d (Ma and Chen, 2020). Polyphenols are commonly found in plants as secondary metabolites, which aid the plant in structural development and react to many biotic and abiotic stressors with well recognized antioxidant activities (Jessica et al., 2019). Our results confirmed that humic acids are a source of antioxidant compounds (Karadirek et al., 2016; Lv et al., 2018). Considering the variability in the phenolic content of microbial-originated products, our results enabled us to identify the properties leonardite.
Although the amount of polyphenols obtained was lower compared with other feed and food products (Castrica et al., 2019), the leonardite antioxidant capacity should be considered high because the phenolic content is comparable to round melon (*Praecitrullus vulgaris*). However its antioxidant capacity is higher than the μmol TroloxEq/g content of lemon and orange water-soluble extracts (Kaur and Kapoor, 2002; Nilsson et al., 2005) which are considered as beneficial foods with positive effects on health. Humic acids can thus be considered as promising biologically-active natural

antioxidants for the development of new classes of pharmaceuticals for medicine (Khil'ko et al., 2011).

Table 2. Chemical composition of diets for control group (CTRL), treatment group (HAG) and leonardite (LEO). All values are expressed as percentage as fed-basis (%).

FA composition (% total FAMEs)

	CTRL	**HAG**
C 8:0	0.78	0.80
C 10:0	0.74	0.81
C 12:0	6.68	7.26
C 14:0	3.82	4.05
C 14:1	0.01	0.02
C 15:0	0.09	0.09
C 16:0	16.36	16.32
C 16:1	0.81	0.81
C 17:0	0.13	0.12
C 17:1	0.06	0.06
C 18:0	4.87	4.85
C 18:1 n9 trans	0.06	0.06
C 18:1 n9 cis	24.15	23.71
C 18:2 n6 cis	35.49	35.18
C 20:0	0.29	0.28
C 18:3 n6	0.04	0.02
C 20:1	0.67	0.63
C 18:3 n3	2.62	2.64
C 21:0	0.02	0.03
C 20:2	0.10	0.14
C 22:0	0.19	0.19
C 20:3 n6	0.01	0.01
C 22:1 n9	0.04	0.04
C 20:3 n3	0.04	0.03
C 20:4 n6	0.11	0.09
C 22:2	0.02	0.02
C 24:0	0.15	0.14
C 20:5 n3	0.64	0.65
C 24:1	0.05	0.04
C 22:6 n3	0.90	0.92

	CTRL	**HAG**	**LEO**
DM	91.62	91.62	81.13
CP	16.24	16.57	6.15
EE	3.79	3.87	0.6
CF	2.69	2.24	5.15
Ashes	4.59	4.46	23.27

DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fiber; FA: fatty acids; FAMEs: fatty acid methyl esters; CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% of leonardite; LEO: leonardite, humic acid-based feed ingredient.

3.3. Trolox Equivalent Antioxidant Capacity (TEAC) of leonardite

Our results showed that the humic acid extract contained a dose-dependent antioxidant capacity (Figure 1; $P < 0.0001$), with a measured amount of 73.31±8.22 μmol TroloxEq/g after 6 minutes. This is in line with other studies on the antioxidant activity of humic substances (Aeschbacher et al., 2012; Smirnova et al., 2012), thus suggesting that the effect is probably related to the content of phenolic moieties as the most important electron-donating groups. In addition, the presence of acid groups (-COOH, -OH) suggests that these substances are capable of an antioxidant effect (Smirnova et al., 2012). The effectiveness of humic substances related to their dose-effect response has also been observed by the gas-volumetric method (Efimova et al., 2012). The results obtained from the latter study showed that leonardite has an antioxidant capacity, highlighting its possible positive effects on animal health by reducing oxidation.

3.4. Zootechnical performance

The final average body weight increased significantly in HAG piglets compared to the CTRL group confirming the positive effect of leonardite treatment on animal growth. The HAG group showed a higher average BW at d 40 compared to CTRL ($P < 0.001$).

Table 3. Results of mineral profile analysed by ICP-MS of two experimental diets (CTRL and HAG diet supplemented with 0.25% leonardite) and leonardite (LEO, humic acids-based feed ingredient). All values are expressed as ppm as fed (mg/kg AF).

	CTRL	**HAG**	**LEO**
Na	1595.72	1697.16	1586.39
Mg	1532.20	1588.64	1524.63
Al	177.23	222.49	8763.21
K	7621.32	8026.69	721.39
Ca	4868.93	4995.59	8309.21
Cr	2.57	2.78	7.69
Mn	68.92	76.08	133.33
Fe	262.84	316.43	6649.14
Co	n.d.	n.d.	3.50
Ni	n.d.	n.d.	6.33
Cu	116.39	98.72	4.52
Zn	133.08	146.15	10.75
As	n.d.	n.d.	4.28
Se	2.40	1.16	0.59
Mo	0.81	0.59	n.d.
Cd	n.d.	n.d.	n.d.
Pb	n.d.	n.d.	5.43
P	6082.46	6339.63	n.d.

n.d. = not detectable

AF: as fed; CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% leonardite; LEO: leonardite, humic acid-based feed ingredient.

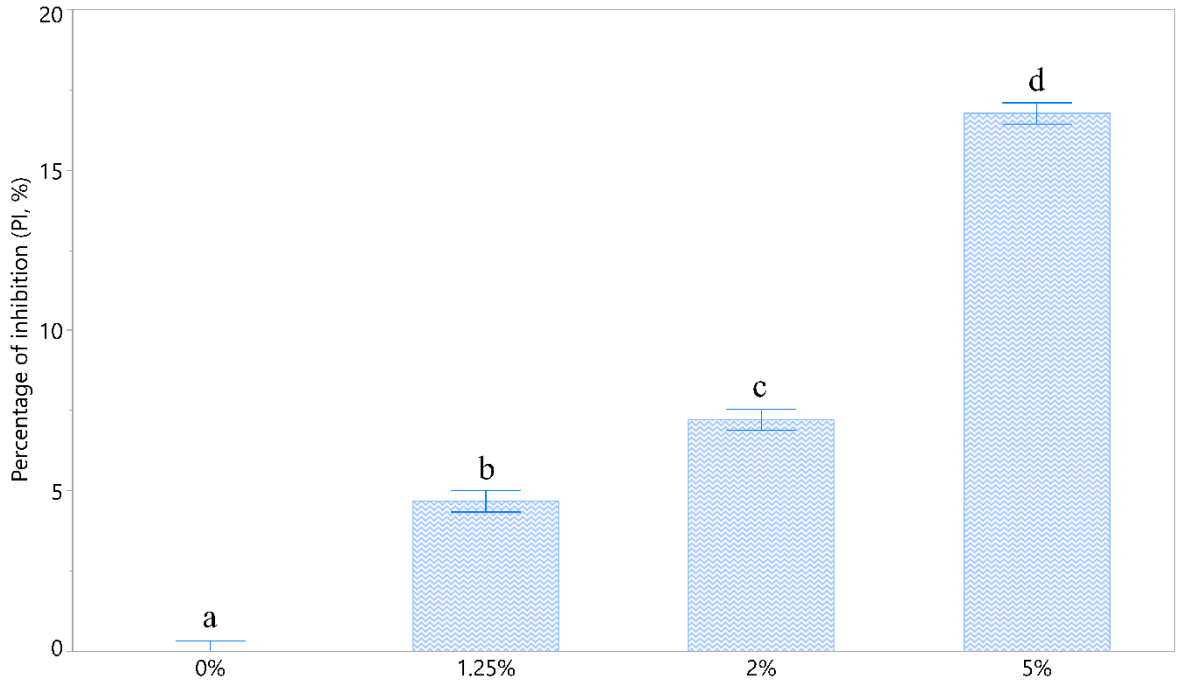

Figure 1. Percentage inhibition of ABTS$^{\bullet+}$ of different concentrations of humic acids extract (blank: 0%; 1.25%; 2% and 5%) measured by Trolox Equivalent Antioxidant Capacity (TEAC) assay.
a-b means with different superscripts are significantly different between treatments ($P < 0.0001$). Data are expressed as least square means (LSMEANS) and Standard Error (SE).
ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

HAG also showed a higher ADG from d 14-28 and from d 28-40 ($P < 0.001$, Table 4). The ADFI of the HAG group increased from d 14-28 and d 28-40 ($P < 0.01$; Table 4) compared to the CTRL group. The higher final body weight of the HAG group was due to a higher consumption of feed although the feed conversion rate did not show significant differences between groups ($P > 0.05$).

Wang et al. (2008) observed a raised ADG with the inclusion of humic substances at 5% and 10% in the diets of the pigs. Trckova et al. (2018) also demonstrated the positive effect of leonardite (supplementation levels of 20 g/kg in the diet) on BW, ADFI and ADG parameters of weaned piglets.

Although it is still not clearly exactly how leonardite exerts its action, the improvements in zootechnical performance seem to be related to the capacity of humic substances to help ion transport through membranes, the protection of intestinal mucosa, the enhancement of enzymes activities, and the better nutrient digestion and adsorption (particularly proteins and minerals) (Trckova et al., 2018).

Table 4. Zootechnical performance of *in vivo* trial (from day 0 to 40) divided by control (CTRL) and treatment (HAG supplemented with 0.25% of leonardite) group.

				P-values		
	CTRL	**HAG**	**SEM±**	Trt	Day	Trt x Day
BW, kg				0.112	< 0.001	< 0.001
d 0	8.71	8.72	0.871			
d 14	11.11	12.21				
d 28	15.44	18.36				
d 40	20.17[a]	24.25[b]				
ADFI, kg/d				0.003	< 0.001	0.254
d 0-14	0.353	0.465	0.034			
d 14-28	0.651[a]	0.841[b]				
d 28-40	0.730[a]	0.891[b]				
ADG, kg/d				< 0.001	< 0.001	0.535
d 0-14	0.171	0.249	0.024			
d 14-28	0.310[a]	0.440[b]				
d 28-40	0.396[a]	0.491[b]				
FCR, kg/kg						
d 0-14	1,97	1,77	0,115	0.384	0.146	0.445
d 14-28	1,93	1,79				
d 28-40	2,03	2,09				

a-b means with different superscripts are significantly different between treatments (P < 0.05).
Data are expressed as least squares means (LSMEANS) and standard error of the mean (SEM).
BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FRC: feed conversion rate; CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% of leonardite.

Bai et al. (2013) estimated that the optimum level for increasing zootechnical performance was a 0.25% supplementation of fulvic acid. Therefore, a low concentration of HA inclusion may improve the growth performance with no influence on the diet composition and feed preparation.

Regarding the health status of the animals throughout the experimental period, only one piglet died at d 7 in the CTRL group. This amounts to a mortality incidence of < 1%, which could be considered as normal in common livestock farming. In both groups, clinical signs of diarrhoea occurred from d 14 to d 21 and was transient (on average lasting three days). The occurrence of diarrhoea (number of new animals with fecal score > 1/total animals per group) was 18.6% and 16.7% in the CTRL and HAG groups at d 14, respectively. The occurrence of diarrhoea was below the average post weaning levels (Carstensen et al., 2005; Laine et al., 2008).

Our results confirmed the increase in BW, ADFI and ADG with a leonardite inclusion of 0.25%, demonstrating that a lower inclusion level could equally enhance the zootechnical performance thus also optimizing the use of leonardite as a feed additive.

3.5. Biological sample analysis

The results of the serum metabolic parameters showed that the HAG group had higher levels of total proteins, albumin, A/G ratio, phosphatase alkaline, glucose, cholesterol, calcium, phosphorous, magnesium, high density lipoprotein, creatinine and total triglycerides ($P < 0.05$, Table 5).

All values were in the physiological range confirming that leonardite did not negatively affect the health status and should be considered as a safe feed additive. An increased albumin and total protein content are related to the age of piglets and their rapid growth (de Meer et al., 2000). The increased A/G ratio was a consequence of the higher albumin value, however no significant difference was observed for the globulin content, whose increase is often related to an inflammatory process (Bertoni et al., 2008).

The ALP content in serum showed a significant difference between the CTRL and HAG ($P < 0.05$) groups. ALP is an important marker of bone remodelling which is involved in cartilage maturation and calcification. ALP in serum is mainly synthetized by liver and bones, and is involved in the formation of phosphorous ions, whose combination with calcium leads to the formation of bone salts (Yuan et al., 2011). The increased ALP level in the HAG group could be explained by a growth burst of the piglets, also connected with a higher level of calcium and phosphorous in serum ($P < 0.05$).

The higher amount of serum magnesium ($P < 0.05$) in the HAG group suggests an enhanced response to stressors. Mg plays a crucial role as an enzymatic cofactor. When the animal is subjected to high levels of stress, catecholamines and stress-associated hormones are released leading to a shift in Mg from the intracellular to the extracellular space. This mechanism increases the urinary excretion of magnesium and subsequent decreases in the serum Mg concentrations. Thus, low serum Mg concentrations increase the release of stress-related hormones, thus establishing a feedback mechanism (Pouteau et al., 2018). Trckova et al. (2018) observed an increased amount of serum Mg, Ca and P in weaned piglets supplemented with leonardite (20 g/kg of diet), thus confirming our findings and suggesting that humic substances could influence the mineral content of serum.

Although the fatty acid profile of the experimental diet was not affected by the inclusion of leonardite, the HAG group exhibited an increased concentration of total cholesterol and decreased triglyceride levels compared with the CTRL group ($P < 0.05$).

Table 5. Metabolic profile analysis of blood serum divided by control (CTRL) and treatment group (HAG supplemented with 0.25% of HA) measured at day 40.

Analyte	CTRL	HAG	SEM±	P-Value
Total protein content, g/L	52.88[a]	61.45[b]	1.33	<0.001
Albumin, g/L	19.31[a]	26.90[b]	1.25	<0.001
Globulin, g/L	33.58	34.57	1.10	0.535
Albumin/Globulin (A/G)	0.58[a]	0.81[b]	0.05	0.002
Urea, mmol/L	2.18	2.30	0.32	0.799
Alanine aminotransferase (ALT-GPT), IU/L	38.33	47.80	3.79	0.093
Aspartate aminotransferase (AST-GOT), IU/L	54.17	50.10	3.32	0.397
Phosphatase alkaline (ALP), IU/L	165.67[a]	228.50[b]	14.41	0.006
Total bilirubin, μmol/L	1.98	2.40	0.24	0.238
Glucose, mmol/L	5.00	5.94	0.35	0.075
Calcium, mmol/L	2.28[a]	2.65[b]	0.06	<0.001
Phosphorus, mmol/L	3.05[a]	3.73[b]	0.09	<0.001
Magnesium, mmol/L	0.85[a]	0.97[b]	0.03	0.014
Creatinine, μmol/L	78.92	90.80	4.43	0.073
Total cholesterol, mmol/L	2.51[a]	2.92[b]	0.12	0.024
High density lipoprotein (HDL), mmol/L	0.77[a]	1.00[b]	0.03	<0.001
Low density lipoprotein (LDL), mmol/L	1.60	1.82	0.09	0.099
Triglycerides, mmol/L	0.68[a]	0.51[b]	0.05	0.027

a-b means with different superscripts are significantly different between treatments ($P < 0.05$).
Data are expressed as least squares means (LSMEANS) and standard error of the mean (SEM).
CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% leonardite.

The increased cholesterol levels could be related to an increased HDL ($P > 0.05$) without any changes in the LDL amount. The increase in HDL may be related to an increased Mg concentration, whose presence or absence is strictly related to the incidence of cardiovascular and metabolic diseases (Verma and Garg, 2017). These results may also be due to the presence of phenolic compounds in leonardite which are recognized by EFSA as important nutrients in protecting blood lipids from oxidative damage and the maintenance of normal blood HDL-cholesterol concentrations without increasing LDL (EFSA, 2011; 2012).

In fact, total cholesterol concentration is an index of the lipometabolic status which includes the free and bounded forms of HDL (Wang et al., 2011). HDL protects blood vessels by decreasing cholesterol levels in the blood stream and guaranteeing their stabilization (Grela and Klebaniuk, 2007). These results suggest that leonardite has a positive effect on the lipidic metabolism as well as protecting blood vessels.

Results from the serum DAO analysis did not show any significant differences between the CTRL (289.40±48.30 ng/mL) and HAG (263.13±33.95 ng/mL) groups. The intact intestinal barrier plays a central role in preventing systemic infection and in the maintenance of the health status. DAO is abundantly expressed in the duodenal and jejunal mucosa and, therefore, DAO activity is a non-invasive marker of alterations in

intestinal mucosal function and structure. Our results are in line with Liu et al. (2016) who showed an improvement in the gut barrier function, decreasing enteric diseases even without significant changes in serum DAO. An increased DAO level is often related to damage of the gut epithelium which releases this enzyme into the blood stream. Thus, the presence of DAO in healthy animals is usually scarce (Hou et al., 2014). Considering no occurrence of diarrhoea during the biological sample collection, although not representing the only indicator of a gut's barrier function, the detected levels of DAO did not reveal any intestinal alteration in either of the experimental groups.

Our RT-PCR data on fecal samples showed a significant reduction in bifidobacteria and lactobacilli in the HAG compared to the CTRL groups. Regarding *E. coli* and Enterobacteriaceae, no difference in abundance was observed.

Based on these preliminary data, it seems that leonardite particularly affects some groups of bacteria. Diarrhoea in piglets is often associated with specific pathogens, however the alteration in the gut microbiota composition can also be involved. In fact, Yang et al. (2017) analysed the gut microbiota of diarrheic neonatal piglets in which no pathogenic *E. coli* were detected. The authors found an increase in *Prevotella* spp. and a reduction in *E. coli* and some beneficial bacteria belonging to the Firmicutes phylum. This altered microbiota led to diarrhoea in neonatal piglets. Yang et al. (2017) results indicate that changes in terms of the relationship between different groups of bacteria can provoke the development of piglet diarrhoea. These microbiota modifications cause the onset of the inflammatory state as well as different nutrient degradation and adsorption abilities. Further analyses are required to investigate the ability of leonardite to modulate the composition of gut microbiota in order to understand the mechanisms of its beneficial effects on piglet performance that we found in our study (Figure 2).

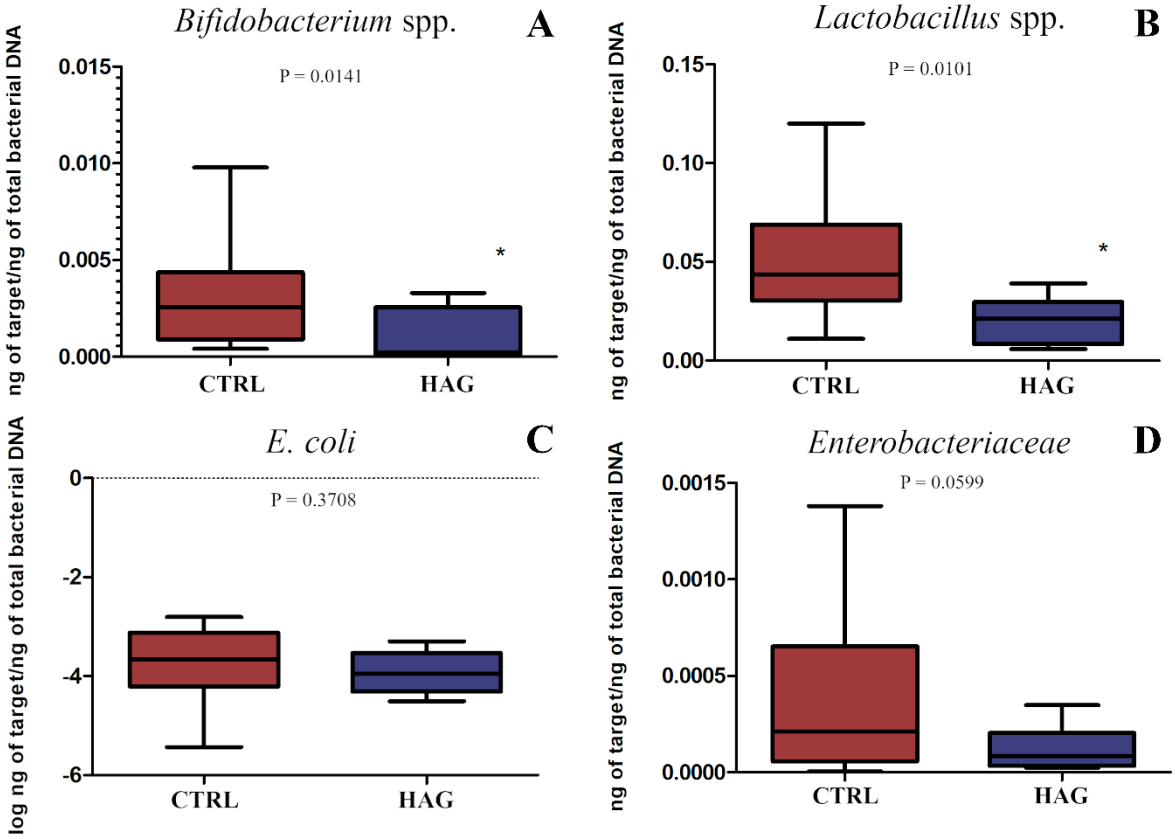

Figure 2. Target DNA of principal microbial indicators of gut microbiota (A: *Bifidobacterium* spp., B: *Lactobacillus* spp., C: *Escherichia coli*, D: *Enterobacteriaceae*) on total bacterial DNA analysed with RT-PCR divided by control and LEO group supplemented with 0.25% leonardite.
*means statistical differences between groups ($P < 0.05$)
CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% leonardite.

Conclusions

Dietary supplementation with 0.25% leonardite improved the zootechnical performance, serum lipidic profile and gut epithelium integrity, thus indicating a good general health status. The increased serum HDL and decreased total triglycerides suggest that leonardite is a promising feed additive to improve lipid metabolism. The higher serum Mg content found also suggests that leonardite supports an improved stress response in weaned piglets.

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Declaration of Competing Interest

The authors declare that no competing interest has interfered with the conduct of the study and the results as presented.

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References

Aeschbacher, M., Graf, C., Schwarzenbach, R., Sander, M., 2012. Antioxidant Properties of Humic Substances. Environmental Science & Technology 46, 4916-4925.

Aksu, T., Bozkurt, A., 2009. Effect of Dietary Essential Oils and/or Humic Acids on Broiler Performance, Microbial Population of Intestinal Content and Antibody Titres in the Summer Season. Kafkas Universitesi Veteriner Fakultesi Dergisi 15, 185-190.

AOAC, 2005. Official Methods of Analysis, Washington, DC.

Attard, E., 2013. A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. Central European Journal of Biology 8, 48-53.

Bai, H.X., Chang, Q.F., Shi, B.M., Shan, A.S., 2013. Effects of fulvic acid on growth performance and meat quality in growing-finishing pigs. Livestock science 158(1-3), 118-123.

Bartosch, S., Fite, A., Macfarlane, G.T., McMurdo, M.E., 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 70, 3575-3581.

Bertoni, G., Trevisi, E., Han, X., Bionaz, M., 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of Dairy Science 91, 3300-3310.

Byun R., Nadkarni M.A., Chhour K.L., Martin F.E., Jacques N.A., Hunter N., 2004. Quantitative analysis of diverse Lactobacillus species present in advanced dental caries. J Clin Microbiol 42, 3128-3136.

Carstensen, L., Ersbøll, A.K., Jensen, K.H., Nielsen, J.P., 2005. Escherichia coli post-weaning diarrhoea occurrence in piglets with monitored exposure to creep feed. Veterinary microbiology 110(1-2), 113-123.

Castrica, M., Rebucci, R., Giromini, C., Tretola, M., Cattaneo, D., Baldi, A., 2019. Total phenolic content and antioxidant capacity of agri-food waste and by-products. Italian Journal of Animal Science 18, 336-341.

Chammui, Y., Sooksamiti, P., Naksata, W., Thiansem, S., Arqueropanyo, O., 2014. Removal of arsenic from aqueous solution by adsorption on Leonardite. Chemical Engineering Journal 240, 202-210.

Christie, W.W., Han, X., 2003. Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids. Third edition, The Oily Press, Bridgwater, England.

Commission Directive 2010/6/EU, amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards mercury, free gossypol, nitrites and Mowrah, Bassia, Madhuca (Text with EEA relevance).

Commission Regulation EU 1275/2013, of 6 December 2013 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic, cadmium, lead, nitrites, volatile mustard oil and harmful botanical impurities (Text with EEA relevance).

Commission Regulation EU 2017/1017, Catalogue of feed materials (Text with EEA relevance.).

Cormican, M., Hopkins, S., Jarlier, V., Reilly, J., Simonsen, G., Strauss, R., et al., 2017. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. Efsa Journal 15.

de Meer, K., Smolders, H., Meesterburrie, J., de Sain-van der Velden, M., Voorbij, H., Okken, A., Reijngoud, D., Kulik, W., 2000. A single food bolus stimulates albumin synthesis in growing piglets. Journal of Pediatric Gastroenterology and Nutrition 31, 251-257.

Dell'Anno, M., Sotira, S., Rebucci, R., Reggi, S., Castiglioni, B., Rossi, L., 2020. *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts. Italian Journal of Animal Science 19(1), 103-113.

Domínguez-Negrete, A., Gómez-Rosales, S., Angeles, M. D. L., López-Hernández, L. H., Reis-de Souza, T. C., López-García, Y., Zavala-Franco, A., Téllez-Isaias, G., 2019. Effect of the Addition of Humic Substances as Growth Promoter in Broiler Chickens Under Two Feeding Regimens. Animals 9(12), 1101, 1-15.

Efimova, I., Khil'ko, S., Smirnova, O., 2012. Antioxidant Activity of Humic Acids in Radical-Chain Oxidation Processes. Russian Journal of Applied Chemistry 85, 1351-1354.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781),"anti-inflammatory properties"(ID 1882),"contributes to the upper respiratory tract health"(ID 3468),"can help to maintain a normal function of gastrointestinal tract"(3779), and "contributes to body defences against external agents" (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal, 9(4), 2033.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012. Scientific Opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006. EFSA Journal, 10(8), 2848.

EGTOP/1/2011, Expert Group for Technical Advice on Organic Production. Final Report on Feed.

Grela, E.R., Klebaniuk, R., 2007. Chemical composition of garlic preparation and its utilization in piglet diets. Medycyna Weterynaryjna 63, 792-795.

Hejna, M., Moscatelli, A., Onelli, E., Baldi, A., Pilu, S., Rossi, L., 2019. Evaluation of concentration of heavy metals in animal rearing system. Italian Journal of Animal Science 18, 1372-1384.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J., Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. Animal Physiology and Animal Nutrition 2, 207-237.

Hou, Y., Wang, L., Yi, D., Ding, B., Chen, X., Wang, Q., Zhu, H., Liu, Y., Yin, Y., Gong, J., Wu, G., 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets

challenged with intrarectal administration of acetic acid. British Journal of Nutrition 111, 1748-1758.
Islam, K.M.S., Schuhmacher, A., Gropp, J.M., 2005. Humic Acid Substances in Animal Agriculture. Pakistan Journal of Nutrition 4, 126-134.
Jessica, P., Amadou, D., Louis-Charles, R., Simon, B., 2019. Bioextracting Polyphenols from the Brown Seaweed Ascophyllum nodosum from Québec's North Shore Coastline. Industrial Biotechnology 15, 212-218.
Ji, F., McGlone, J., Kim, S., 2006. Effects of dietary humic substances on pig growth performance, carcass characteristics, and ammonia emission. Journal of Animal Science 84, 2482-2490.
Kaevska, M., Lorencova, A., Videnska, P., Sedlar, K., Provaznik, I., Trckova, M., 2016. Effect of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on faecal microbiota composition in weaned piglets. Veterinarni Medicina 61, 328-336.
Karadirek, Ş., Kanmaz, N., Balta, Z., Demirçivi, P., Üzer, A., Hızal, J., Apak, R., 2016. Determination of total antioxidant capacity of humic acids using CUPRAC, Folin–Ciocalteu, noble metal nanoparticle- and solid–liquid extraction-based methods. Talanta 153, 120-129.
Kaur, C., Kapoor, H.C., 2002. Anti-oxidant activity and total phenolic content of some Asian vegetables. International Journal of Food Science & Technology 37(2), 153-161.
Khil'ko, S., Efimova, I., Smirnova, O., 2011. Antioxidant properties of humic acids from brown coal. Solid Fuel Chemistry 45, 367-371.
Kim, K., Bae, I., Cho, J., Choi, Y., Ha, J., Choi, J., 2019. Effects of Humic Acid and Blueberry Leaf Powder Supplementation in Feeds on the Productivity, Blood and Meat Quality of Finishing Pigs. Food Science of Animal Resources 39, 276-285.
Laine, T.M., Lyytikäinen, T., Yliaho, M., Anttila, M., 2008. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. Acta Veterinaria Scandinavica 50(21), 1-11.
Liu, G., Guan, G., Fang, J., Martinez, Y., Chen, S., Bin, P., Duraipandiyan, V., Gong, T., Tossou, M., Al-Dhabi, N., Yin, Y., 2016. Macleaya cordata Extract Decreased Diarrhea Score and Enhanced Intestinal Barrier Function in Growing Piglets. Biomed Research International 1-7.
Lv, J., Han, R., Huang, Z., Luo, L., Cao, D., Zhang, S., 2018. Relationship between Molecular Components and Reducing Capacities of Humic Substances. Acs Earth and Space Chemistry 2, 330-339.
Ma, G., Chen, Y., 2020. Polyphenol supplementation benefits human health via gut microbiota: A systematic review via meta-analysis. Journal of Functional Foods 66, 103829, 1-11.
Mennen, L.I., Walker, R., Bennetau-Pelissero, C., Scalbert, A., 2005. Risks and safety of polyphenol consumption. The American Journal of Clinical Nutrition 81(1), 326S–329S.
Nilsson, J., Pillai, D., Önning, G., Persson, C., Nilsson, Å., Åkesson, B., 2005. Comparison of the 2, 2′-azinobis-3-ethylbenzotiazo-line-6-sulfonic acid (ABTS) and ferric reducing anti-oxidant power (FRAP) methods to asses the total antioxidant capacity in extracts of fruit and vegetables. Molecular nutrition & food research 49(3), 239-246.
NRC, 2012. Nutrient Requirements of Swine. National Research Council, The National Academies Press, Washington, DC.
Ozturk, E., Ocak, N., Turan, A., Erener, G., Altop, A., Cankaya, S., 2012. Performance, carcass, gastrointestinal tract and meat quality traits, and selected blood parameters of broilers

fed diets supplemented with humic substances. Journal of the Science of Food and Agriculture 92(1), 59-65.

Patrone, V., Minuti, A., Lizier, M., Miragoli, F., Lucchini, F., Trevisi, E., Rossi, F., Callegari, M.L., 2018. Differential effects of coconut versus soy oil on gut microbiota composition and predicted metabolic function in adult mice. BMC Genomics 19(1), 808, 1-17.

Penders, J., Vink, C., Driessen, C., London, N., Thijs, C., Stobberingh, E.E., 2005. Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol Lett. 243, 141-147.

Pouteau, E., Kabir-Ahmadi, M., Noah, L., Mazur, A., Dye, L., Hellhammer, J., Pickering, G., Dubray, C., 2018. Superiority of magnesium and vitamin B6 over magnesium alone on severe stress in healthy adults with low magnesemia: A randomized, single-blind clinical trial. Plos One 13(12), 1-17.

Prior, R., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53, 4290-4302.

Regulation EC 1831/2003, of the European Parliament and of the council, of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance).

Rossi, L., Dell'Orto, V., Vagni, S., Sala, V., Reggi, S., Baldi, A., 2014. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic Escherichia coli strain in piglets. Veterinary Research Communications 38, 39-49.

Rossi, L., Vagni, S., Polidori, C., Alborali, G.L., Baldi, A., Dell'Orto, V., 2012. Experimental Induction of E*scherichia coli* Diarrhoea in Weaned Piglets. Open Journal of Veterinary Medicine 2, 1-8.

Smirnova, O., Efimova, I., Khil'ko, S., 2012. Antioxidant and Pro-oxidant Activity of Ascorbic and Humic Acids in Radical-Chain Oxidation Processes. Russian Journal of Applied Chemistry 85, 252-255.

Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2018. The effect of leonardite and lignite on the health of weaned piglets. Research in Veterinary Science 119, 134-142.

Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2017. Effects of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on the health, oxidative stress status and fatty acid profile in weaned piglets. Veterinární medicína 62(1), 16-28.

Trckova, M., Lorencova, A., Hazova, K., Zajacova, Z., 2015. Prophylaxis of post-weaning diarrhoea in piglets by zinc oxide and sodium humate. Veterinarni Medicina 60, 351-360.

Verma, H., Garg, R., 2017. Effect of magnesium supplementation on type 2 diabetes associated cardiovascular risk factors: a systematic review and meta-analysis. Journal of human nutrition and dietetics 30(5), 621-633.

Wang, J., Wu, C., Feng, J., 2011. Effect of dietary antibacterial peptide and zinc-methionine on performance and serum biochemical parameters in piglets. Czech Journal of Animal Science 56, 30-36.

Wang, Q., Chen, Y., Yoo, J., Kim, H., Cho, J., Kim, I., 2008. Effects of supplemental humic substances on growth performance, blood characteristics and meat quality in finishing pigs. Livestock Science 117, 270-274.

Yang, Q., Huang, X., Zhao, S., Sun, W., Yan, Z., Wang, P., Li, S., Huang, W., Zhang, S., Liu, L., Gun, S., 2017. Structure and Function of the Fecal Microbiota in Diarrheic Neonatal Piglets. Front Microbiol. 8(502), 1-13.

Yuan, X., Wang, J., Zhu, X., Zhang, Z., Ai, Y., Sun, G., Wang, Z., Liu, G., 2011. Effect of Copper on Levels of Collagen and Alkaline Phosphatase Activity from Chondrocytes in Newborn Piglets *In Vitro*. Biological Trace Element Research 144, 597-605.

Zhang, J., Lv, Y., Tang, C., Wang, X., 2013. Effects of dietary supplementation with palygorskite on intestinal integrity in weaned piglets. Applied Clay Science 86, 185-189.

Zhao, J., Harper, A.F., Estienne, M.J., Webb Jr, K.E., McElroy, A.P., Denbow, D.M., 2007. Growth performance and intestinal morphology responses in early weaned pigs to supplementation of antibiotic-free diets with an organic copper complex and spray-dried plasma protein in sanitary and nonsanitary environments. Journal of Animal Science 85(5), 1302-1310.

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Evaluation of Tannin Extracts, Leonardite and Tributyrin Supplementation on Diarrhoea Incidence and Gut Microbiota of Weaned Piglets

Matteo Dell'Anno [1,*], Serena Reggi [1], Valentina Caprarulo [2], Monika Hejna [1], Carlo Angelo Sgoifo Rossi [1], Maria Luisa Callegari [3], Antonella Baldi [1] and Luciana Rossi [1]

Brief Introduction to the Study:

The following study was selected as 'editor's choice' by the scientific editors of MDPI's journal Animals as one of the best recently published articles of particular interest to readers and for the importance of its contribution to the field of research. The study proposes an innovative combination of feed additives never tested before, as result of previous publications that evaluated each compound individually, on the reduction of diarrhoea and gut microbiota modulation in weaned piglets. The following work was a conclusive study of a cycle of functional compounds evaluation that involved the assessment of tannin extracts, leonardite and tributyrin as alternatives to antibiotics. The results showed that the innovative mix of functional compounds significantly reduced the incidence of diarrhoea, increased the lactic acid:coliform bacteria ratio, decreased blood low-density lipoprotein (LDL) and modulated the gut microbiota. The article contributed to the scientific knowledge on alternatives to antibiotics for reducing antibiotic resistance and increasing the sustainability of pig farming.

4.5 Evaluation of Tannin Extracts, Leonardite and Tributyrin Supplementation on Diarrhoea Incidence and Gut Microbiota of Weaned Piglets

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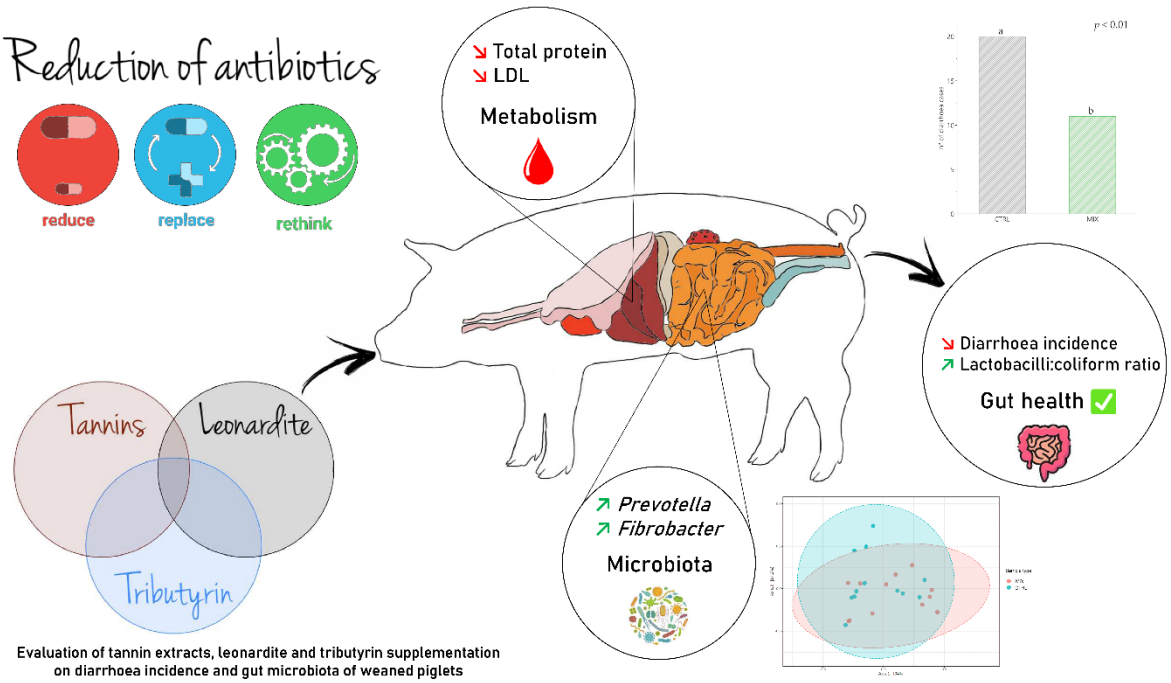
Simple Summary: Replacing and reducing antimicrobial treatments in livestock farming has become crucial for animal, human and environmental health due to global concerns regarding antimicrobial resistance. Weaning is a stressful phase in which piglets can develop enteric disorders leading to the massive use of antibiotics. In this scenario, functional nutrition represents one alternative to reducing antimicrobial resistance. The aim of this study was to assess the effects of the dietary administration of the combination of tannin extracts, leonardite and tributyrin supplementation on weaned piglets. After weaning, piglets were divided into two experimental groups: one fed a basal diet (CTRL) and one fed a basal diet supplemented with 0.75% Quebracho and Chestnut tannin extracts, 0.25% leonardite and 0.20% tributyrin (MIX), respectively, for 28 days. Zootechnical performance and diarrhoea incidence were recorded. Microbiological and microbiota analyses were performed on faecal samples, and metabolic profile of blood serum was evaluated. The MIX group revealed a reduced incidence of diarrhoea and improved faecal consistency compared to the CTRL group. After 28 days, MIX revealed an increased lactobacilli:coliform ratio compared to CTRL, and the serum metabolic profile showed lower levels of low-density lipoproteins, suggesting a modulation of the lipid metabolism. The results suggest that the combination of tannin extracts, leonardite and tributyrin could improve animal health, thus reducing diarrhoea occurrence in weaned piglets.

Graphical abstract:



Abstract: The effects of the dietary administration of a combination of Quebracho and Chestnut tannins, leonardite and tributyrin were evaluated in weaned piglets. A total of 168 weaned piglets (Landrace × Large White) were randomly allotted to two experimental groups (6 pens/group, 14 piglets/pen). Animals were fed a basal control diet (CTRL) and a treatment diet (MIX) supplemented with 0.75% tannin extracts, 0.25% leonardite and 0.20% tributyrin for 28 days. Individual body weight and feed intake were recorded weekly. Diarrhoea incidence was recorded by a faecal scoring scale (0–3; considering diarrhoea ≥ 2). At 0 and 28 days, faecal samples were obtained from four piglets/pen for microbiological and chemical analyses of faecal microbiota, which were then assessed by V3-V4 region amplification sequencing. At 28 days, blood from two piglets/pen was sampled to evaluate the serum metabolic profile. After 28 days, a reduction in diarrhoea incidence was observed in the MIX compared to CTRL group ($p < 0.05$). In addition, compared to CTRL, MIX showed a higher lactobacilli:coliform ratio and increased *Prevotella* and *Fibrobacter* genera presence ($p < 0.01$). The serum metabolic profile showed a decreased level of low-density lipoproteins in the treated group ($p < 0.05$). In conclusion, a combination of tannin extract, leonardite and tributyrin could decrease diarrhoea incidence and modulate the gut microbiota.

Keywords: alternatives to antibiotics; antimicrobial resistance; feed additives; functional feed; tannins; leonardite; tributyrin; microbiota; diarrhoea incidence; weaned piglets

1. Introduction

In livestock, alternatives to antibiotics that are capable of promoting the health status, preventing diseases and reducing medical treatments are needed in order to tackle increasing global antibiotic resistance [1,2]. Although it is not totally clear how antibiotic use in food-producing animals spreads resistant bacteria in humans, replacing antimicrobials is a key aim of European policies [3,4].

This need was particularly highlighted by the removal of zinc oxide licensed as a medicinal product as it is involved in environmental pollution and in the co-selection of antibiotic-resistant bacteria [5,6]. In fact, following the ban on the use of antibiotics as growth promoters, therapeutic antibiotics and zinc oxide have become more widely used [7–9] to prevent porcine colibacillosis, improve suboptimal weight gain, and feed efficiency. Alternatives to antibiotics and zinc oxide are also urgently needed to guarantee the profitability of swine farming, particularly during weaning, which involves the largest use of antimicrobials due to the high incidence of gastroenteric disorders and multifactorial post-weaning diarrhoea (PWD) [10,11]. The gastrointestinal tract (GIT) is a highly specialised organ where the dynamic interaction between host cells and the complex environment (mucosal chemical barrier, immune system, and epithelium) impacts on gut health [12]. Gut health is important in the reduction of diseases and the optimal functioning of digestive processes, along with optimal production performance.

The aim of nutrition is no longer simply to satisfy nutritional requirements; it also plays a key role in the health and welfare of humans and animals [13]. The nutritional components of animal feed are thus continually adjusted to optimize the effects on animal health and growth. Functional feed ingredients, which sustain the health status and reduce the risk of pathologies, have thus become fundamental in replacing or reducing antimicrobials in food-producing animals [14,15]. Studies have thus focused on developing nutraceutical alternatives to antibiotics in order to maintain swine health and performance [16]. In addition to their nutritional value, functional feed ingredients contain bioactive compounds and nutricines which exert beneficial activities on the organism (immunomodulatory, antioxidant, anti-inflammatory, antibacterial effects, etc.) with positive impacts also on animal performance and general farm profitability [17–19].

Tannins have antioxidant, anti-inflammatory and antimicrobial properties, and are used to enhance growth performance, modulate intestinal microbiota, and decrease the incidence of diarrhoea, particularly during the post-weaning period [20] through their antimicrobial and cytomodulatory effects on intestinal cells [21].

Hydrolysable and condensed tannins increase performance and animal gut health, and reduce diarrhoea by directly inhibiting enterotoxigenic *Escherichia coli* bacteria when supplemented in weaned piglets [22]. They disrupt the bacterial wall by releasing hydrogen peroxide from their hydroxyl groups [23]. In addition, tannins may reduce cortisol levels in animals and protect against lipid peroxidation, leading to a decreased levels of plasma malondialdehyde [24].

Leonardite is rich in humic acids, and due to its macrocolloid structure can protect intestinal mucosa by reducing the resorption of toxic metabolites from the residues of harmful substances in feed [25]. Humic acids prevent an excessive loss of water from the gut, which is important in the treatment of diarrhoea, dyspepsia, and acute intoxications [26]. In weaned piglets, leonardite improves animal performance and modulates lipid metabolism by increasing the level of HDL cholesterol in blood serum and suggesting an enhanced defence from stressors through a higher Mg serum level after 40 days of supplementation [27]. Leonardite seems to act by helping ion transport through membranes, protecting intestinal mucosa, enhancing enzymatic activities and promoting nutrient digestion and adsorption, particularly for proteins and minerals [28].

Some specific dietary short chain and medium chain fatty acids play a key role in the intestinal inflammation of pigs, as well as in modulating the intestinal microbial population and in promoting digestion [29]. The supplementation of 0.20% of tributyrin was shown to boost animal performance, lipid metabolism and gut health through increased energy metabolism of enteric bacteria and promoting the richness positively related to animal performance and mucosal immune function [30]. Tributyrin can act as a histone deacetylase inhibitor (HDAC), stimulating muscle growth through satellite cell differentiation in muscular tissue promoting animal performance [31]. In addition, butyrate supplementation may increase villus height and crypt depth in the duodenum [32]. Antibacterial activity related to butyrate was observed through a reduction in intestinal pH and a decrease in harmful bacteria in the caecum [33].

Despite the high number of products studied as alternatives to antimicrobials in commercialised feed for swine farming, few studies have specifically investigated the synergistic or antagonistic effects of possible additive combinations on the health and performance of weaned piglets. Due to encouraging results obtained in our previous studies from the supplementation of single additives, the aim of this study was to assess the possible combined effects of Quebracho and Chestnut tannin extracts, leonardite and tributyrin supplementation on animal health and microbiota modulation in weaned piglets.

2. Materials and Methods

2.1. Animals, Housing, Experimental Design and Treatment

The experimental trial was approved by the Animal Welfare Organization of the University of Milan (OPBA authorization no. 09/2020) and performed in accordance with European regulations [34]. The trial was conducted on a commercial farm that was free from pathologies included in the ex-list A of the World Organization for Animal Health (Porcine Reproductive Respiratory Syndrome, atrophic rhinitis, transmissible gastroenteritis, salmonellosis and Aujeszky disease).

The study lasted 28 days, and included 168 weaned piglets (Landrace x Large White; 28 ± 2 days) homogeneous by gender (50% male, 50% female) and weight (7.48 ± 1.16 kg). Piglets were identified by individual ear tags and housed in 12 different pens (14 animals/pen), in standardised environmental conditions (27 °C and 60% relative humidity).

After an adaptation period of three days with the same basal diet, piglets were allotted to a randomised complete block design in two experimental groups: control group (CTRL: 84 piglets, 6 pens) fed the basal diet (ad libitum), and the treatment group (MIX, 84 piglets, 6 pens) fed the basal diet (ad libitum) supplemented with 0.75% Quebracho and Chestnut tannin extracts (Silvafeed® Nutri P, Silvateam, Mondovì, Italy), 0.25% leonardite (New Feed Team, Lodi, Italy) and 0.20% tributyrin (ACIFIS® Tri-B, New Feed Team, Italy), based on previous studies [20,27,30]. The two experimental isoproteic and isoenergetic diets (Table 1) were balanced using Plurimix System® software v. 2.4 (Fabermatica, Cremona, Italy) in order to meet the nutritional requirements for post-weaned piglets [35] and were provided by Ferraroni S.p.A. (Cremona, Italy). Considering the small inclusion percentage, the additives were premixed with wheat flour to ensure a homogeneous dispersion before being added to the horizontal mixer with the other ingredients, substituting 2% of wheat meal with 2% of the experimental mix (0.80% wheat flour 00, 0.75% tannin extracts, 0.25% leonardite, and 0.20% tributyrin).

Table 1. Diet composition and principal chemical characteristics of *in vivo* trial (% as fed basis) divided by control (CTRL, fed basal diet) and treatment group (MIX, fed basal diet supplemented with 0.75% Quebracho and Chestnut Tannin extract, 0.25% leonardite and 0.20% tributyrin).

Ingredients, % as Fed Basis	**CTRL**	**MIX**
Barley, meal	26.84	26.84
Wheat, meal	12.45	10.45
Corn, flakes	11.63	11.63
Corn, meal	10.00	10.00
Barley, flakes	7.50	7.50
Soy protein concentrates	5.00	5.00
Biscuits, meal	4.00	4.00
Soybean, meal (44%)	4.00	4.00
Dextrose monohydrate	3.50	3.50
Sweet milk whey	2.50	2.50
Herring, meal	2.00	2.00
Plasma, meal	2.00	2.00
Beet pulp	1.40	1.40
Acidifiers [1]	1.70	1.70
Coconut oil	1.00	1.00
Soy oil	1.00	1.00
Dicalcium phosphate	0.60	0.60
L-Lysine	0.60	0.60
Benzoic acid	0.50	0.50
Vitamins and mineral premix [2]	0.50	0.50
DL-Methionine	0.39	0.39
L-Threonine	0.35	0.35
Sodium Chloride	0.27	0.27
L-Valine (96.5%)	0.12	0.12
Enzyme mix [3]	0.10	0.10
L-Tryptophan	0.05	0.05
Experimental mix [4]	-	2.00
Calculated Chemical Composition [5]		
Crude protein (%)	18.65	18.33
Fat (%)	4.78	4.75
Crude fibre (%)	3.00	2.98
Ashes (%)	5.52	5.48
DE [6] (Mc/Kg)	3.92	3.83

[1] Citric acid, fumaric acid, orthophosphoric acid, sorbic acid, calcium formate. [2] Additives per Kg: Vitamins, pro-vitamins and substances with similar effect. Retinyl Acetate 15000 IU, Vitamin D3-Cholecalciferol 2000 IU, Vitamin E 120 mg, Vitamin B1 2.0 mg, Vitamin B2 4.8 mg, Vitamin B6 3.4 mg, Calcium D-pantothenate 15.0 mg, Vitamin B12 0.030 mg, Vitamin K3 1.9 mg, Biotin 0.19 mg, Niacinamide 30.0 mg, Folic Acid 0.96 mg, Vitamin C 144 mg, Choline chloride 288 mg, Betaine hydrochloride 1000 mg, Compounds of trace elements Iron sulphate 115 mg, Manganese Oxide 48.0 mg, Zinc Oxide 96.1 mg, Copper Oxide 130 mg, Anhydrous Calcium Iodate 0.96 mg, Sodium Selenite 0.34 mg. [3] 6-phytase, endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase. [4] The experimental mix was composed of 0.80% wheat flour 00 and the three supplemented additives: 0.75% Quebracho and Chestnut tannin extracts (Silvafeed® Nutri P, Silvateam, Mondovì, Italy), 0.25% leonardite (New Feed Team, Lodi, Italy), 0.20% tributyrin (ACIFIS® Tri-B, New Feed Team, Lodi, Italy). [5] Calculation performed with Purimix System® software (Fabermatica, Cremona, Italy). [6] DE: digestible energy content estimated from NRC (2012).

2.2. Chemical Evaluation of Experimental Diets

The experimental diets were analysed in duplicate in terms of principal nutrients: dry matter (DM), ether extract (EE), crude protein (CP), crude fibre (CF), and ash content. Dry matter (DM) was obtained by drying samples in a forced air oven at 65 °C for 24 h (AOAC method 930.15). CP was determined by the Kjeldahl method (AOAC method 2001.11). EE was determined using ether extraction in the Soxtec system (DM 21/12/1998). CF was determined by the filtering bag technique [36]. Ash content was obtained by incinerating samples in a muffle furnace at 550 °C (AOAC method 942.05).

2.3. Zootechnical Performance, Diarrhoea Incidence and Sampling Procedures

Body weight (BW) was individually recorded at days 0 (T0), 7 (T1), 14 (T2), 21 (T3) and 28 (T4). Feed intake was recorded weekly for each pen by measuring the feed refused, considering the pen as the experimental unit. Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated. For the microbiological and microbiota analyses, faecal samples were collected at T0 and at T4 from the rectal ampulla of four piglets per pen randomly selected (24 piglets CTRL, 24 piglets MIX).

Faecal consistency of the four selected piglets was scored on a weekly basis using a four-level scale: 0 = normal consistency (faeces firm and well formed); 1 = soft consistency (faeces soft and formed); 2 = mild diarrhoea (fluid faeces, usually yellowish); 3 = severe diarrhoea (faeces watery and projectile). A faecal consistency score ≤ 1 (0,1) was considered normal, whereas a faecal score >1 (2,3) was defined as diarrhoea. Faecal colour was also evaluated using a three-level scale: 1 = yellowish colour; 2 = greenish colour; 3 = brown colour. A faecal colour ≥ 2 (greenish-brown) was considered normal, while a faecal colour < 2 (yellowish) was considered pathological [11].

Blood was sampled from the jugular vein of two randomly selected subjects per pen at T0 and T4, using vacuum tubes without any anticoagulant.

2.4. Blood Serum Analysis

Serum samples were obtained by centrifugation, and were analysed using a multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA) at 37 °C. We measured the following concentration of: total protein (g/L), albumin (g/L), globulin (g/L), albumin/globulin (A/G ratio), alanine aminotransferase (ALT-GPT; IU/L), glucose (mmol/L), urea (mmol/L), creatinine (μmol/L), total bilirubin (μmol/L), total cholesterol (mmol/L),

triglycerides (mmol/L), high-density lipoprotein (HDL; mmol/L), low-density lipoprotein (LDL; mmol/L), phosphorus (mmol/L), and magnesium (mmol/L).

Serum glucagon and insulin concentrations were also quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for pigs according to the manufacturer's instructions (Mecordia Inc, Uppsala, Sweden; Cusabio Technology Llc, Houston, Texas). Absorbances were measured with a microplate reader at 450 nm (Bio-Rad 680 microplate reader, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and concentrations were calculated according to the respective standard curve using CurveExpert v. 1.4.

2.5. Microbiological Analysis of Faecal Samples

The faecal samples were analysed in terms of the total bacteria (Plate Count Agar, PCA), lactobacilli (De Man, Rogosa and Sharpe Agar, MRS) and coliform count (Violet Red Bile Broth Agar, VRBA). Briefly, 1 g of each faecal sample was homogenised with 10 mL of sterile physiological solution and centrifuged (3000 rpm for 10 min) to collect the supernatant. Samples were serially diluted, and microorganisms were enumerated by plate counting after 24 h of semi-anaerobic incubation at 37 °C using the overlay method for MRS and VRBA, and the inclusion method for PCA [37–39]. The lactobacilli:coliform ratio was calculated based on plate counting results which were expressed as log10 of colony-forming units per gram of faeces (log_{10} CFU/g).

2.6. Nitrogen Content, Apparent Nitrogen Digestibility, Volatile Fatty Acids and pH of Faecal Samples

Faeces collected at 28 days were dried in a forced-air oven and analysed for nitrogen content (AOAC method 930.15; AOAC method 2001.11). Apparent nitrogen digestibility was assessed through the acid insoluble ash (AIA) marker [40] after incinerating feed and faecal samples in a muffle furnace at 550 °C (AOAC method 942.05). Apparent nitrogen digestibility was then calculated using the following equation:

$$Apparent\ Nutrient\ Digestibility\ (\%) = 100 \times \left(1 - \frac{marker\ in\ feed}{marker\ in\ feaces} \times \frac{nutrient\ in\ faeces}{nutrient\ in\ feed}\right) \quad (1)$$

The fresh faecal sample pH of T4, diluted in 10 mL of sterile physiological solution and subsequently centrifuged, were measured for the supernatant using a pH meter.

Volatile fatty acid analysis was performed by gas chromatography (GC), and the samples were prepared as follows: 0.5 gof faecal samples were dissolved in 1 mL of distilled water and thoroughly mixed for a few minutes. Following centrifugation (10.000 g for 10 min at 10°C), 0.5 mL of supernatant was added to 250 µL of oxalic acid (0.12 M) and 250 µL of pivalic acid solution (1 g pivalic acid + 50 mL formic acid, filled to 1 L with distilled water). After mixing and centrifugation (10.000 g for 10 min at 10°C), the clear supernatant was transferred into the vial, and injected into the GC. Volatile fatty acids (VFA) were quantified according to Ahmed et al. [41].

2.7. Bacterial DNA Extraction, V3-V4 Region Amplification and Sequencing

Faecal samples were collected at 28 days and stored in frozen dry ice until further processing. Bacterial DNAs were extracted starting with 50 mg (fresh weight) of faecal sample using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Eschwege, Switzerland) according to the manufacturer's instructions. Extracted DNA was quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA), whereas the DNA quality check was carried out through agarose gel electrophoresis. DNA was sent to Fasteris SA (Geneva, Switzerland) for sequencing. The amplicons were sequenced by Illumina MiSeq v. 3 in 2 × 300 bp mode. Trimmomatics v. 0.32 (http://www.usadellab.org/cms/index.php?page = trimmomatic, accessed on 26/08/2020) [42] was used to remove the adapter sequence from the reads, and during this process, the filtering was performed. Filtering was performed by SLIDINGWINDOW, a sliding window trimming, which cuts the read tail when four consecutive bases are of low quality. By considering multiple bases, a single poor quality base was thus not the cause of the removal of high quality data later in the read (window size: 4 base, quality: 15). Filtering was also performed by MINLEN which drops the read when it is below a specified length (set at 60 bases).

Filtered reads were mapped against the SILVA database using Burrows–Wheeler Alignment Tool v. 0.7.5a (http://bio-bwa.sourceforge.net/, accessed on 28/08/2020). SAM tools was used to merge alignments and to compute the number of reads onto each OTU. Sequence files are available in the European Nucleotide Archive (ENA) database under accession number ID PRJEB43937.

2.8. Statistical Analysis

The results were analysed using a generalised linear model that assesses the leverage of the effects based on the analysis of variances using JMP 14 Pro® (SAS Inst. Inc., Cary, NC, USA). For animal performance, faecal bacterial counts, the model included the fixed effect of treatments (Trt), the effect of time (Time), and the interaction between treatment and time (Trt × Time). Serum metabolites data were

evaluated after performing analysis of covariances (ANCOVA) to adjust the initial variability of serum samples. Data on diarrhoea incidence were assessed using Pearson's Chi-Squared test. The results from faecal nitrogen content, immunoenzymatic kits, apparent digestibility, volatile fatty acids and faecal pH at T4 were analysed using ANOVA. Pearson correlations were performed. Multiple comparisons between groups were evaluated with Tukey's Honestly Significant Difference test (Tukey's HSD). Results were presented as least square means ± standard error (SE). Means were considered different when $p \leq 0.05$.

All statistical analyses concerning sequences obtained were performed using MicrobiomeAnalyst [43,44], which calculates alpha diversity based on Chao 1, Observed species, Simpson and Shannon metrics. Significant differences in these indices were calculated using a t test ANOVA. Beta diversity across samples was instead calculated using the Bray–Curtis index and PERMANOVA statistical methods. The beta diversity across the microbial community of animals belonging to both dietary groups were visualised using a PCoA plot. The edgeR algorithm with 0.05 adjusted *p*-value cut-off was used to identify significant differences in taxa abundance between the two groups of animal microbiota. The Linear discriminant analysis Effect Size (LEfSe) Sparse Correlations for Compositional data (SparCC) and Random Forest analysis were performed using the same tool.

GraphPad Prism v. 8 (GraphPad Prism, San Diego, CA, USA) was used to perform the t test and Spearman's correlation analysis, respectively.

3. Results and Discussion

3.1. Chemical Evaluation of the Experimental Diets

Nutrient profile of both experimental diets was in line with NRC [35] guidelines fulfilling the nutritional requirements of post-weaning piglets. The inclusion in the diet of 0.75% tannin extracts, 0.25% leonardite and 0.20% tributyrin did not influence the main nutrient profiles of MIX group experimental diet (Table S1).

3.2. Zootechnical Performance

Zootechnical performance, in swine farming, besides being the main concern for farmers, is considered as an indirect indicator of intestinal health. Both groups revealed a progressive increase in body weight in line with standard growth curves, ADG and ADFI (Table S2). No detrimental effects of the combination of ingredients were observed.

The results showed no significant differences in BW, ADG, ADFI, FCR between the experimental groups. The effect on the zootechnical performance could well have

been more exacerbated with longer experimental periods [27,30]. In fact, for those additives affecting animal production or performance, long-term efficacy and safety studies are necessary, which correspond to periods of 42 days in post-weaning piglets [45,46]. In addition, the positive effects of functional and antioxidant dietary compounds are more evident when animals have a pathological condition (e.g., experiential infection, intestinal injuries) [47,48], whereas in this study the animals were in good general health throughout the experimental period.

3.3. Diarrhoea Incidence

Diarrhoea is one of the main issues in the weaning phase in swine farming and represents the most evident sign of dysbiosis. On the other hand eubiosis, an indicator of gut health, is ensured through a positive interaction between the host, the microorganisms and the environment. At the beginning of the trial, piglets were healthy without any signs of diarrhoea (T0). The highest numbers of diarrhoea were during the first 14 days (17 piglets with faecal score ≥ 2) in both groups. The first two weeks of weaning are considered as the most critical phase for diarrhoea incidence, which is caused by maternal immunity reduction and the impact of stressors, leading to decreased performance and antibiotic use [49]. There was a higher incidence of diarrhoea in the CTRL group (20 cases; 16.67% of faeces evaluated) compared with the MIX group (11 cases; 9.17% of faeces evaluated) throughout the experimental period ($p < 0.01$; Figure 1). Regarding faecal colour, a yellowish colour at 7 days was recorded in only four animals (1 CTRL and 3 MIX) with no statistical differences at any point during experimental period. Post-weaning diarrhoea is recognised as a multifactorial disease. It can be influenced by post-weaning fasting, together with environmental and feeding stress. From a microbiological point of view, many factors could be involved in the aetiology, such as bacteria, parasites and viruses [50].

Although the individual effects of each functional component supplemented cannot be differentiated, their combination lowered the occurrence of diarrhoea. Previous results obtained in our studies showed that all three compounds tested separately had a positive effect on gut health. In this study, due to their different mechanisms of action these compounds may also have contributed to the reduction in diarrhoea. In fact, Chestnut and Quebracho tannins are antimicrobial and antioxidant substances with a powerful effect on enterotoxigenic *Escherichia coli*, which are the main pathogens involved in diarrhoea occurrence in weaned piglets [51,52]. Tannins may directly affect bacterial growth, impairing the bacterial cell wall and indirectly supporting the antioxidant status of animals [23,24]. In addition, the inclusion of leonardite in the feed, characterised by a large amount of humic substances, likely stabilizes the microbial intestinal population, improving intestinal barrier health and

preventing diarrhoea. Although the effect of leonardite, is still not fully understood, it could be due to the affinity of humic substances to biological membranes and their participation in ion transportation, which may boost performance and health status [27,28].

Humic acids contained in leonardite have shown an ability to lower pH in the gastrointestinal tract and stabilize intestinal flora [53]. Tributyrin also reduces diarrhoea due to its nutrient absorption, and the intestinal morphology of villi enhancement in weaned piglets [54]. Tributyrin and butyric acid lower the pH of GIT (particularly in the stomach and intestine) promoting an increase in beneficial bacteria [32].

The decrease in diarrhoea incidence and the positive effect on faecal consistency could be related to the in-feed supplementation of the mixture of functional compounds. These results are in line with other results showing a reduction in diarrhoea related to the inclusion of tannin extracts, leonardite and tributyrin individually supplemented in animal diets, and proposed as a valuable alternative to antibiotics for weaned piglets [28,55–57]. Intestinal integrity supported by tributyrin has been widely demonstrated in the literature, due to the release of three butyric acid molecules in the gut. The effects of lowering the pH and strengthening epithelium integrity could increase animal resistance to diarrhoea [33,57]. Each additive in the mixture is characterised by a different composition and mechanism of action. Hence, our results suggest that they could improve intestinal health through a dynamic interaction with the GIT environment.

Although numerous feed additives have been proposed in pig diets, there are contrasting results in the literature. Establishing one additive as an alternative to antibiotics in the feed is therefore not possible; however, since no antibiotics are used as growth promoters, some functional compounds will be beneficial when fed to pigs [58].

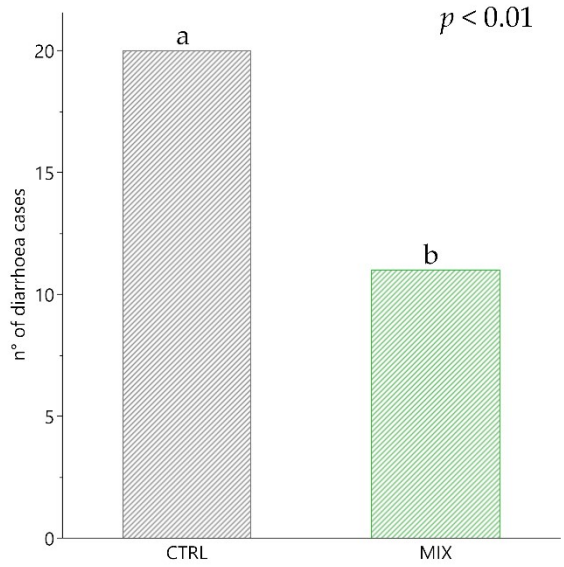


Figure 1. Diarrhoea incidence from 0 to 28 days of experimental period divided per control (CTRL) and treatment group (MIX). [a–b] Means with different superscripts are significantly different between treatments ($p < 0.05$). CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extracts, 0.25% leonardite and 0.20% tributyrin in the diet.

3.4. Serum Metabolic Profile

The results from the serum metabolic profile revealed biochemical levels in line with reference values and with previously obtained data from single additive supplementation in both groups (Table 2) [20,27,30,59–61], thus confirming the absence of toxic effects on the main serum metabolic parameters. However, a significant reduction in LDL cholesterol was observed in the MIX group compared to the CTRL group (1.21 ± 0.08 and 1.48 ± 0.07, respectively; $p < 0.05$). Low-density lipoproteins are well known for their risk factors related to the development of circulatory diseases. Blood concentrations of LDL promote atherosclerosis and cardiovascular diseases [62] and low LDL serum levels are key for cardiovascular prevention and treatment [63].

Tributyrin can modulate lipid metabolism since short chain fatty acids can lower LDL cholesterol levels [64]. These particular forms of three esterified fatty acids have been associated with a low phosphorylated c-JUN-NH2 terminal kinase content with partial hepatic steatosis reversion, leading to a reduction in fat accumulation [65]. In our study, the decrease in LDL cholesterol level confirmed the encouraging results on the lipid metabolism modulation of leonardite and tributyrin supplementation, positively affecting the animal health status [27,30].

The MIX group revealed a statistically significant lower total protein content in blood serum compared to CTRL (47.75 ± 1.67 and 54.92 ± 1.46 g/L, respectively; $p < 0.05$). Tannins are able to establish stable and insoluble complexes with dietary

proteins [66,67], which could slightly reduce the protein bioaccessibility in the gut, leading to a decrease in serum total protein. Although tannins could potentially reduce protein digestibility, we found no side effects related to animal performance, thus suggesting that the reduction did not impair animal performance and health, in line with Caprarulo et al. [20]. The blood serum urea level revealed a tendency to decrease in the MIX compared to the CTRL group (0.78 ± 0.07 and 0.99 ± 0.06, respectively; $p < 0.09$). Serum urea is a nonprotein nitrogen directly associated with CP concentration in the feed. Circulating urea could be considered a useful indicator for diet formulation and nitrogen use [68]. Caprarulo et al. [20] highlighted a shift in protein metabolism due to tannin supplementation after 40 days, leading to an increased bacterial protein synthesis.

Table 2. Metabolic profile of blood serum divided by control (CTRL) and treatment group (MIX) measured at day 28.

Serum Metabolite	CTRL	MIX	SE CTRL	SE MIX	*p*-Value
Total protein content, g/L	54.92 [a]	47.75 [b]	1.46	1.67	0.0258
Albumin, g/L	27.89	25.91	1.24	1.42	0.3808
Globulin, g/L	25.85	23.31	1.44	1.62	0.2938
Albumin/Globulin (A/G)	1.06	1.18	0.06	0.07	0.2465
Urea, mmol/L	0.99	0.78	0.06	0.07	0.0746
Alanine aminotransferase (ALT-GPT), IU/L	54.75	56.81	4.68	5.27	0.7867
Total bilirubin, µmol/L	1.90	1.80	0.11	0.13	0.5750
Glucose, mmol/L	6.19	6.91	0.48	0.55	0.4169
Phosphorus, mmol/L	3.26	3.10	0.13	0.15	0.4547
Magnesium, mmol/L	0.92	0.88	0.04	0.05	0.6328
Creatinine, µmol/L	70.18	65.27	3.81	4.47	0.5098
Total cholesterol, mmol/L	2.62	2.36	0.12	0.13	0.1901
High density lipoprotein (HDL), mmol/L	1.01	1.04	0.07	0.08	0.7955
Low density lipoprotein (LDL), mmol/L	1.48 [a]	1.21 [b]	0.07	0.08	0.0435
Triglycerides, mmol/L	0.60	0.69	0.09	0.10	0.4982
Insulin, mU/L	10.78	9.47	2.57	2.87	0.7437
Glucagon, pg/mL	294.10	286.51	13.99	22.38	0.8103

[a–b]Means with different superscripts are significantly different between treatments ($p < 0.05$). Data are expressed as least square means (LSMEANS) and standard errors (SE). CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extracts, 0.25% leonardite and 0.20% tributyrin in the diet.

3.5. Microbiological Analysis of Faecal Samples

Total plate counting, coliform and lactobacilli faecal content revealed no differences between groups at T0 and T4, highlighting a similar content in total bacteria and in coliform and lactobacilli in piglets' faeces (Figure 2). T0 was characterised by a high prevalence of lactobacilli (5.81 ± 0.20 $\log_{10}$ CFU/g for CTRL and 5.85 ± 0.21 $\log_{10}$ CFU/g for MIX). On the other hand, T4 showed a statistically

significant reduction of this group of bacteria (4.23 ± 0.21 $\log_{10}$ CFU/g for CTRL and 4.49 ± 0.21 $\log_{10}$ CFU/g for MIX; $p < 0.0001$).

Before weaning, lactobacilli or lactic acid bacteria are usually higher in piglets due to the milk consumption, and may decrease naturally after weaning and feeding with solid diets [69]. In our study, the lactobacilli:coliform bacteria ratio increased at day 28 (T4) in the MIX compared to CTRL group (1.79 ± 0.13 and 1.20 ± 0.13 $\log_{10}$ CFU/$\log_{10}$ CFU, respectively; $p < 0.01$). The lactobacilli:coliform ratio predicts intestinal health and is used in efficacy tests of feed additives and acidifiers in order to promote immune defence. After weaning, the ratio changes depending on the level of coliform and immune defence development by the host. The lactobacilli:enterobacteria ratio is adopted as a simple index whose increase is related to a higher resistance to intestinal disorders [70].

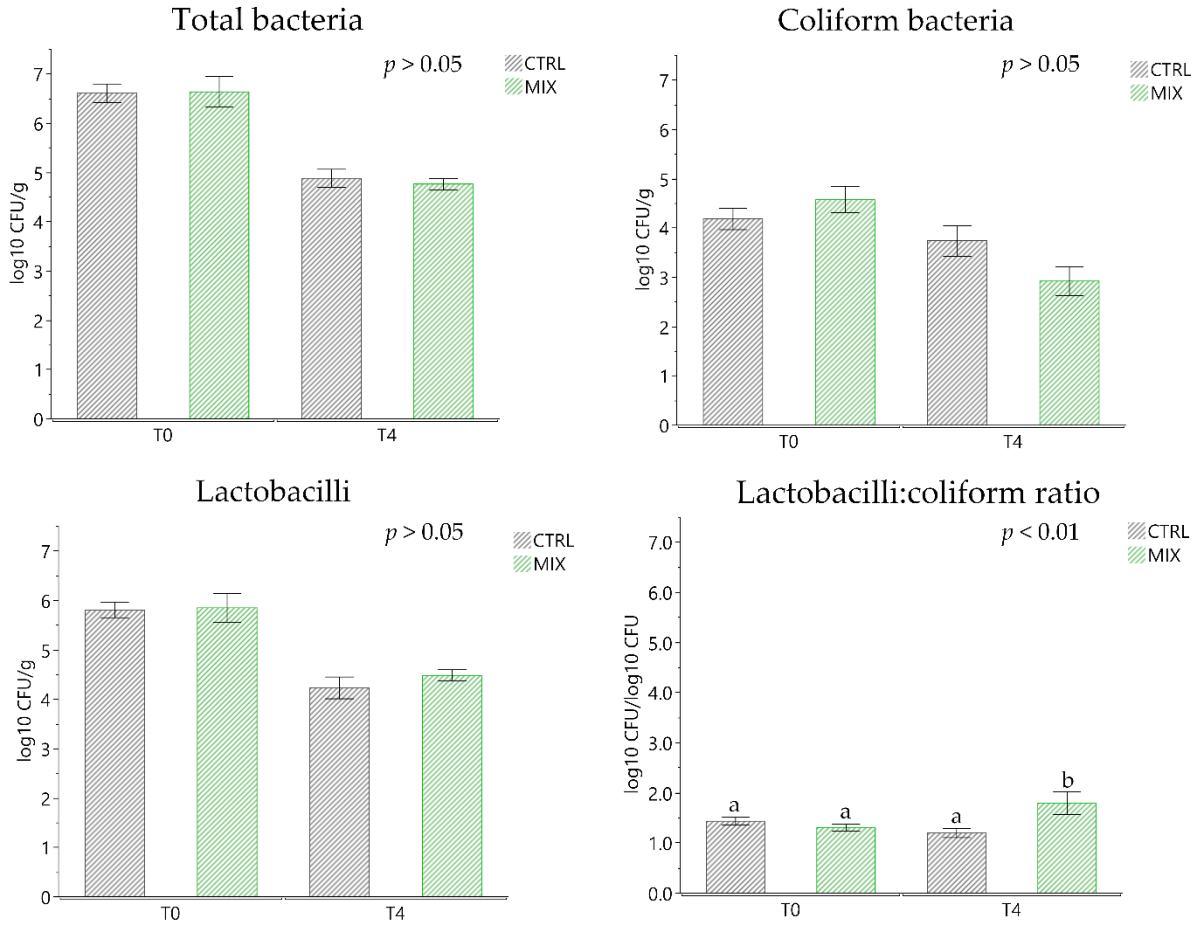


Figure 2. Faecal content of the principal bacterial groups (total bacteria, coliform, lactobacilli and lactobacilli:coliform ratio) divided by control (CTRL) and treatment (MIX) at the beginning (T0) and after 28 days of trial (T4). [a–b] Means with different superscripts are significantly different between treatments ($p < 0.05$). Data are expressed as least square means (LSMEANS) and standard errors (SE). CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extract, 0.25% leonardite and 0.20% tributyrin in the diet.

3.6. Nitrogen Content, Apparent Nitrogen Digestibility, Volatile Fatty Acids and pH of Faecal Samples

The faecal nitrogen content revealed a statistically significant increase in the MIX compared to the CTRL group (4.19 ± 0.10 and 3.85 ± 0.10% on dry matter basis, respectively; $p < 0.05$) at T4. A linear positive correlation (r = 0.79; $p < 0.0001$) was observed for the faecal nitrogen content (fresh weight basis) and dry matter of faeces, highlighting that more solid faeces had a higher nitrogen concentration. The effects of tannins on decreasing protein bioavailability and forming indigestible complexes are well known [71]. The results suggest that the tannin extract supplemented in the MIX group diet increased the nitrogen excretion. However, this effect did not influence the animal growth performance, as confirmed by a non-statistically significant difference in the nitrogen excretion between the two experimental groups, considering nitrogen percentages on a fresh weight basis (1.13 ± 0.05% for CTRL and 1.24 ± 0.05% for MIX group).

Although an increased nitrogen output suggests a lower protein availability, which is key for piglet growth, the performance showed no differences in pig growth. This was confirmed by the apparent nitrogen digestibility which revealed no statistically significant difference between the CTRL (80.96 ± 7.37%) and MIX (83.12 ± 6.75%) group. The availability of the nitrogen in the diet was thus not affected by the dietary treatment. These results are in line with Caprarulo et al. [20] who, after supplementing tannin extract in the feed, observed a shift in protein metabolism, which immediately promoted bacterial growth in the large intestine, which were able to exploit undigested substrate. No significant differences in faecal VFA content were detected between the two experimental groups (Table S3), suggesting that the microbial fermentations of the MIX group were not influenced by the treatment.

The faecal pH measured at T0 and at T4 showed no statistically significant differences between the CTRL and MIX (7.00 ± 0.08 and 7.01 ± 0.08, respectively) groups, thus highlighting that the inclusion of the experimental mix in the animals' diet did not influence this parameter. Faecal pH is a cheap method that provides important information on intestinal health. Faecal pH values over 8.5 could indicate ammonia formation from intestinal fermentation caused by decreased protein digestion [72].

The results suggest that neither leonardite (rich in organic acids) tannins (protein-binding ability) nor tributyrin (intestinal pH lowering effect) significantly impaired protein digestion, intestinal fermentation, and faecal pH.

3.7. Microbiota Composition and Community Diversity Associated with Mix Supplementation.

A total of 264,055 filtered sequences were obtained in the samples collected from the MIX and CTRL groups. The median sequencing coverage was 52,668 sequences per sample. The beta-diversity was evaluated based on the PERMANOVA analysis of the Bray–Curtis distances. Samples were not clustered separately, although PERMANOVA analysis indicated significant differences between the two dietetic groups (R2 = 0.087, $p < 0.038$) (Figure 3). In addition, the MIX supplementation had no significant influence on the faecal bacterial alpha diversity calculated by Chao 1 ($p = 0.29$), observed OTUs ($p = 0.55$), Simpson ($p = 0.86$) and Shannon ($p = 0.93$) indices. As a consequence, no significant differences in species diversity and richness were observed among dietary groups.

At the phylum level, the faecal microbiota collected from the control group were dominated by *Firmicutes* (89%) followed by *Bacteroidetes* (7.5%), whereas in animals receiving the MIX supplementation, *Firmicutes* represented 83% and *Bacteroidetes* 14%. The presence of *Actinobacteria* phylum was less than 1% in both groups. These differences between the two groups were significant for both *Firmicutes* (FDR = 0.023) and *Bacteroidetes* (FDR < 0.0001). The decrease in *Actinobacteria* was also significant (FDR = 0.023) in the treated group of animals. Among the less abundant phyla in the MIX group, *Fibrobacteres* was significantly higher (FDR < 0.001), whereas *Chlamydiae* and *Cyanobacteria* were significantly lower than the control group (Table 3).

At the family level, *Prevotellaceae* and *Fibrobacteraceae* increased significantly in samples collected from the MIX group, whereas *Chlamydiaceae* decreased ($p < 0.01$; Table 4). Additionally, at the family level, weak correlations were detected. The *Fibrobacteriaceae* and *Prevotellaceae* families correlated positively with ADFI (r = 0.480, $p = 0.018$; and r = 0.563, $p = 0.004$, respectively) and FCR (r = 0.477, $p = 0.018$; r = 0.561, $p = 0.004$, respectively).

At the genus level, a significant increase in *Prevotella* and *Fibrobacter* was detected in the piglets' supplemented diet, whereas the relative abundance of RFN20, *Eubacterium*, *Lachnospira*, *Desulfovibrio* and *Chlamydia* was low ($p < 0.01$). The values of fold changes and FDR are reported in Table 4. The FCR of *Fibrobacter* (r = 0.477, $p = 0.018$) and *Prevotella* (r = 0.573, $p = 0.03$) showed very similar correlation values to those obtained with ADFI. *Fibrobacter* and *Prevotella* are well-known dietary fibres that degrade bacteria and produce short chain fatty acids [73].

The genus *Chlamydia* is a well-known cause of disease. Within the genus, several species have been identified and in particular, in pigs, *Chlamydia suis* seems to be widespread and related to the presence of other pathogens [74]. In weaned piglets,

Chlamydia spp. has been associated with intestinal microscopic lesions in healthy [75] as well as in diarrhoeic animals [76].

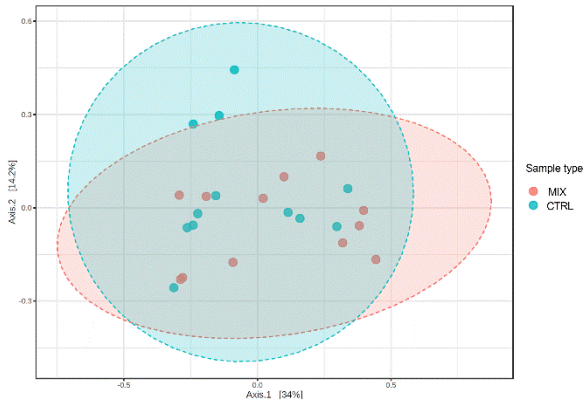


Figure 3. Principal coordinate analysis (PCoA, Bray–Curtis distance) plot of the gut microbiota of weaned piglets fed a diet with (MIX) or without mix supplementation (CTRL). CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extract, 0.25% leonardite and 0.20% tributyrin in the diet.

Table 3. Differentially abundant phyla between MIX and CTRL groups of piglets.

Phylum	log2FC	Log CPM	*p*-Values	FDR
Bacteroidetes	1.973	17.166	<0.0001	<0.0001
Fibrobacteres	2.736	11.433	<0.0001	0.0002
Chlamydiae	−2.860	10.731	0.0058	0.0192
Actinobacteria	−1.449	6.976	0.0101	0.0238
Firmicutes	1.119	20.203	0.0119	0.0238
Cyanobacteria	−1.566	7.227	0.0274	0.0456
Verrucomicrobia	−2.050	11.582	0.0356	0.0509

CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extract, 0.25% leonardite and 0.20% tributyrin in the diet.

Table 4. Differentially abundant families and genera between MIX fed animals and CTRL group.

Family	log2FC	Log CPM	*p* Values	FDR
Prevotellaceae	2.010	16.721	<0.0001	<0.0001
Fibrobacteraceae	2.359	11.288	0.0004	0.0053
Chlamydiaceae	−3.149	10.902	0.0035	0.0326
Genus				
Prevotella	1.963	16.848	<0.0001	0.0003
RFN20	−2.295	7.274	<0.0001	0.0003
Eubacterium	−4.652	14.923	<0.0001	0.0003
Fibrobacter	2.396	11.328	0.0006	0.0047
Lachnospira	−2.050	12.780	0.0032	0.0214
Desulfovibrio	−2.301	8.221	0.0081	0.0443
Chlamydia	−2.701	10.827	0.0107	0.0503

CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extract, 0.25% leonardite and 0.20% tributyrin in the diet.

LEfSe analysis revealed that the two dietary groups could be differentiated at the family level by *Desulfovibrionaceae*, *Coriobacteriaceae* and *Prevotellaceae* (Figure 4A). The

first two families were more abundant in the CTRL group, whereas *Coriobacteriaceae* family was more abundant in the MIX group.

At the genus level, *Lachnospira*, RFN20, *Desulfovibrio* and *Bulleidia* characterised the gut microbiota of the CTRL group, whereas *Prevotella* showed a very high LDA score associated with MIX group samples (Figure 4B). These results were confirmed by the random forest analysis which indicated *Desulfovibrionaceae*, *Coriobacteriaceae* and *Prevotellaceae* families as differentiating the two dietary groups. The discriminant genera resulting from the previous analysis were in agreement with those of the random forest analysis (Figure 5A and Figure 5B).

All these analyses thus confirmed that the MIX supplementation reduced *Desulfovibrionaceae* and *Coriobacteriaceae,* whereas it increased the abundance of *Prevotellaceae*. In the gut, *Coriobacteriaceae* have been associated with bile salts and steroid conversion. In addition, they have shown particular characteristics involved in the conversion of food polyphenols [77]. In humans, the family *Desulfovibrionaceae* and in particular the *Desulfovibrio* genus, have been associated with the inflammation status, as well as being involved in the disruption of the intestinal barrier [78]. In piglets, *Desulfovibrionaceae* increase during the weaning period and play a crucial role in H_2 balance, thus maintaining suitable conditions for intestinal fermentation [79].

Concerning *Prevotellaceae*, contradictory results have been reported, in particular regarding their impact on pig performance. Negative correlations between animal body weight and abundance of *Prevotellaceae* have been described [80], while the *Prevotella*-dominant enterotype has been associated with higher feed intake values compared to the *Treponema*-dominant enterotype in Duroc pigs [81]. As suggested by Amat et al. [82], further analyses are required to clarify the role of specific species of *Prevotella* in pig performance. Within this family, *Prevotella copri* is the most abundant species found in pig gut microbiota after weaning. This species is present during the nursing period, increases at weaning, remains very abundant in the growth phases and decreases in the finishing phase [83]. The link between *Prevotella* species and the development of diarrhoea is still controversial. In fact, in some conditions, its high abundance has been associated with a preventive effect on diarrhoea development [84], while in other studies, the high presence of *Prevotella* spp. has been posited as promoting this disease [81]. Based on our results, the increased level of the *Prevotella* genus was not associated with a high faecal score or with the frequency of diarrhoea episodes.

Finally, genera co-occurrence network analysis revealed relationships between *Prevotella* and *Lachnospira*, RFN20, *Desulfovibrio*, *Bacteroides*, *Eubacterium* and *Bulleidia*. The estimation of sparse correlations revealed a negative correlation between *Prevotella* and *Lachnospira* (SparCC = −0.67; $p < 0.01$), RFN20 (SparCC = −0.92; $p < 0.01$),

Desulfovibrio (SparCC = −0.54; $p = 0.03$) and *Bulleidia* (SparCC = −0.62; $p = 0.03$) genera. These results indicate an antagonistic relationship between *Prevotella*, which was increased in the MIX-diet group, and bacterial genera which were, on the contrary, reduced due to the dietary treatment. Quebracho and Chestnut tannin extracts, leonardite and tributyrin supplementation seemed mainly to increase the genus *Prevotella*, which seems to modulate other microorganisms. Although the role of *Prevotella* has not yet been completely defined, in our study its presence was correlated with ADFI and not with diarrhoea. Further studies are needed to clarify the effects on the gut microbiota of this microbial genus.

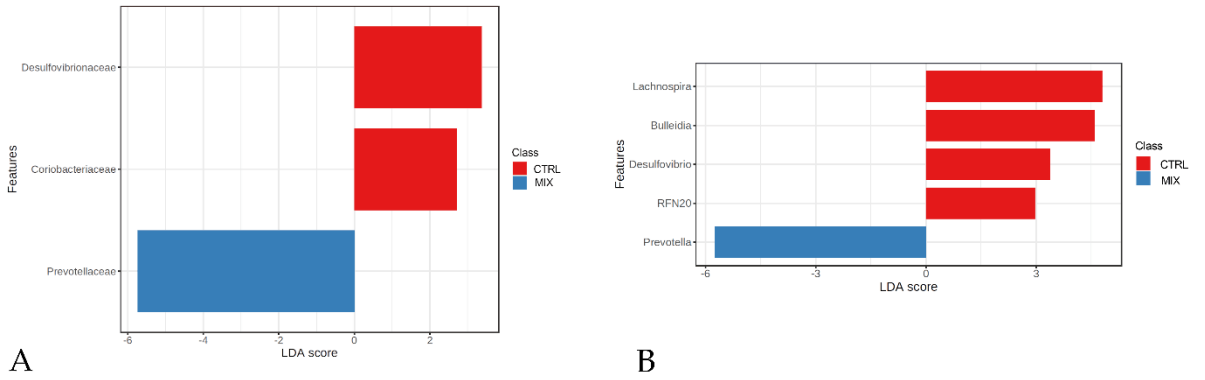


Figure 4. LEfSe analysis results between control (CTRL) and mix group (MIX) of animals at the family (**A**) and the genus level (**B**). CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extract, 0.25% leonardite and 0.20% tributyrin in the diet.

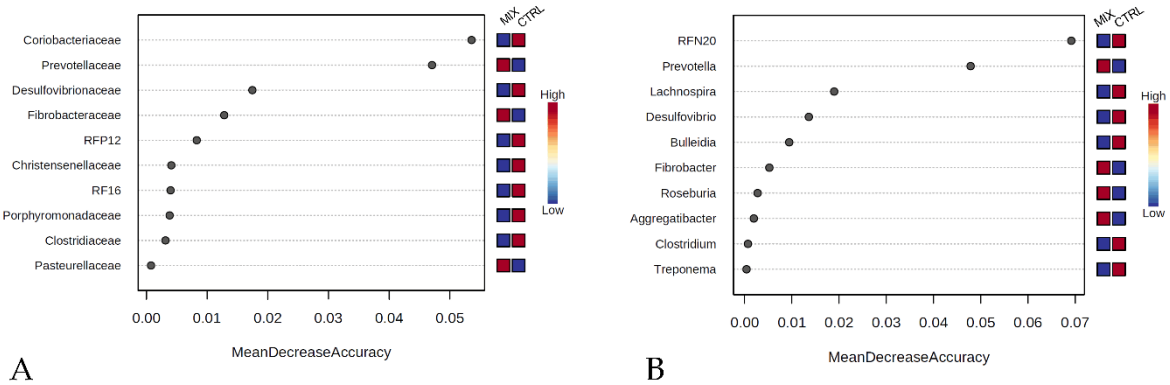


Figure 5. Random forest analysis results between mix fed animals (MIX) and control group (CTRL) at the family (**A**) and at the genus level (**B**) CTRL: control group; MIX: treatment group supplemented with 0.75% tannin extract, 0.25% leonardite and 0.20% tributyrin in the diet.

4. Conclusions

We found that the dietary administration of a combination of Chestnut and Quebracho tannin extracts, leonardite and tributyrin to significantly reduce the occurrence of diarrhoea and increase the lactobacilli:coliform ratio after 28 days, thus promoting animal health. Functional compound supplementation also revealed the positive regulation of lipid metabolism, thus confirming the possible role of tributyrin

and leonardite in modulating the fatty acid profile in blood serum. Our results indicated that this supplementation promotes changes to gut microbial communities, particularly increasing *Prevotella* spp. In conclusion, the in-feed supplementation of Quebracho and Chestnut tannin extracts, leonardite and tributyrin could be a promising alternative for the judicious use of antimicrobials in weaned piglets, which is considered a global sustainability priority. However, further studies are needed to better clarify the exact mechanism of action and the optimal concentration of these three functional compounds to maximise their effect on animal health and performance.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11061693/s1, Table S1: Chemical composition of experimental diets: CTRL (basal diet) and MIX (basal diet supplemented with 0.75% of Quebracho and Chestnut tannin extracts, 0.25% of leonardite, 0.20% of tributyrin), Table S2: Zootechnical performance of the experimental trial (from day 0 to 28) divided by control (CTRL) and treatment (MIX) group, Table S3: Mean values of faecal VFA proportion of tannin extracts, leonardite and tributyrin supplementation (MIX) and control (CTRL) groups.

Author Contributions: Conceptualization, M.D. and L.R.; methodology, M.D., S.R. and L.R.; formal analysis, M.D. and M.L.C.; investigation: M.D., S.R., M.L.C., V.C. and M.H.; data curation: M.D., M.L.C.; writing—original draft preparation, M.D.; writing—review and editing: M.D., M.L.C., M.H. and L.R.; visualization: M.D., M.L.C.; supervision: L.R., A.B. and C.A.S.R.; project administration: L.R.; funding acquisition: L.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and the experimental trial was approved by the AnimalWelfare Organization of University of Milan (OPBA authorization n_ 09/2020).

Data Availability Statement: The data presented in this study are available within the article and Supplementary Materials.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Arsène, M.M.; Davares, A.K.; Andreevna, S.L.; Vladimirovich, E.A.; Carime, B.Z.; Marouf, R.; Khelifi, I. The use of probiotics in animal feeding for safe production and as potential alternatives to antibiotics. *Vet. World* **2021**, *14*, 319.
2. Ng, W.-J.; Shit, C.-S.; Ee, K.-Y.; Chai, T.-T. Plant Natural Products for Mitigation of Antibiotic Resistance. In *Sustainable Agriculture Reviews 49*; Springer: Berlin/Heidelberg, Germany, 2021; pp. 57–91.
3. Tang, K.L.; Caffrey, N.P.; Nóbrega, D.B.; Cork, S.C.; Ronksley, P.E.; Barkema, H.W.; Polachek, A.J.; Ganshorn, H.; Sharma, N.; Kellner, J.D. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: A systematic review and meta-analysis. *Lancet Planet. Health* **2017**, *1*, e316–e327.
4. Cormican, M.; Hopkins, S.; Jarlier, V.; Reilly, J.; Simonsen, G.; Strauss, R.; Vandenberg, O.; Zabicka, D.; Zarb, P.; Catchpole, M.; et al. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. *EFSA J.* **2017**, *15*, doi:10.2903/j.efsa.2017.5017.
5. European Medicines Agency (EMA). Questions and Answers on Veterinary Medicinal Products Containing Zinc Oxide to be Administered Orally to Food-Producing Species. In *Outcome of a Referral Procedure under Article 35 of Directive 2001/82/EC (EMEA/V/A/118)*; EMA/394961/2017; European Medicines Agency: London, UK, 2017.
6. Hejna, M.; Onelli, E.; Moscatelli, A.; Bellotto, M.; Cristiani, C.; Stroppa, N.; Rossi, L. Heavy-Metal Phytoremediation from Livestock Wastewater and Exploitation of Exhausted Biomass. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2239.
7. European Parliament. *Regulation No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition*; Official Journal of the European Union,: Brussels, Belgium, 2003.
8. Bonetti, A.; Tugnoli, B.; Piva, A.; Grilli, E. Towards Zero Zinc Oxide: Feeding Strategies to Manage Post-Weaning Diarrhea in Piglets. *Animals* **2021**, *11*, 642.
9. Hejna, M.; Moscatelli, A.; Onelli, E.; Baldi, A.; Pilu, S.; Rossi, L. Evaluation of concentration of heavy metals in animal rearing system. *Ital. J. Anim. Sci.* **2019**, *18*, 1372–1384, doi:10.1080/1828051X.2019.1642806.
10. Lu, C.W.; Wang, S.E.; Wu, W.J.; Su, L.Y.; Wang, C.H.; Wang, P.H.; Wu, C.H. Alternative antibiotic feed additives alleviate pneumonia with inhibiting ACE-2 expression in the respiratory system of piglets. *Food Sci. Nutr.* **2021**, *9*, 1112–1120.
11. Rossi, L.; Dell'Orto, V.; Vagni, S.; Sala, V.; Reggi, S.; Baldi, A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. *Vet. Res. Commun.* **2014**, *38*, 39–49, doi:10.1007/s11259-013-9583-9.

12. Chelakkot, C.; Ghim, J.; Ryu, S.H. Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp. Mol. Med.* **2018**, *50*, 1–9.
13. Domínguez Díaz, L.; Fernández-Ruiz, V.; Cámara, M. The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 1738–1746.
14. Lallès, J.-P.; Montoya, C.A. Dietary alternatives to in-feed antibiotics, gut barrier function and inflammation in piglets post-weaning: Where are we now? *Anim. Feed Sci. Technol.* **2021**, 114836.
15. Rossi, C.; Compiani, R.; Baldi, G.; Muraro, M.; Marden, J.; Rossi, R.; Pastorelli, G.; Corino, C.; Dell'Orto, V. Organic selenium supplementation improves growth parameters, immune and antioxidant status of newly received beef cattle. *J. Anim. Feed Sci.* **2017**, *26*, 100–108, doi:10.22358/jafs/70765/2017.
16. Tan, B.; Lim, T.; Boontiam, W. Effect of dietary supplementation with essential oils and a Bacillus probiotic on growth performance, diarrhoea and blood metabolites in weaned pigs. *Anim. Prod. Sci.* **2020**, *61*, 64–71.
17. Alemayehu, T.A.; Geremew, A.; Getahun, A. The Role of Functional Feed Additives in Tilapia Nutrition. *Fish Aquac. J.* **2018**, *9*, g1.
18. Bi, Y.; Yang, C.; Diao, Q.; Tu, Y. Effects of dietary supplementation with two alternatives to antibiotics on intestinal microbiota of preweaned calves challenged with *Escherichia coli* K99. *Sci. Rep.* **2017**, *7*, 1–12.
19. Dell'Anno, M.; Sotira, S.; Rebucci, R.; Reggi, S.; Castiglioni, B.; Rossi, L. *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts. *Ital. J. Anim. Sci.* **2020**, *19*, 103–113, doi:10.1080/1828051X.2019.1703563.
20. Caprarulo, V.; Hejna, M.; Giromini, C.; Liu, Y.; Dell'Anno, M.; Sotira, S.; Reggi, S.; Sgoifo-Rossi, C.A.; Callegari, M.L.; Rossi, L. Evaluation of Dietary Administration of Chestnut and Quebracho Tannins on Growth, Serum Metabolites and Fecal Parameters of Weaned Piglets. *Animals* **2020**, *10*, 1945.
21. Reggi, S.; Giromini, C.; Dell'Anno, M.; Baldi, A.; Rebucci, R.; Rossi, L. *In Vitro* Digestion of Chestnut and Quebracho Tannin Extracts: Antimicrobial Effect, Antioxidant Capacity and Cytomodulatory Activity in Swine Intestinal IPEC-J2 Cells. *Animals* **2020**, *10*, 195.
22. Caprarulo, V.; Giromini, C.; Rossi, L. Chestnut and quebracho tannins in pig nutrition: The effects on performance and intestinal health. *Animal* **2020**, 100064, doi:10.1016/j.animal.2020.100064.
23. Wang, Y.; Xu, Z.; Bach, S.; McAllister, T. Sensitivity of *Escherichia coli* to seaweed (*Ascophyllum nodosum*) phlorotannins and terrestrial tannins. *Asian Australas. J. Anim. Sci.* **2009**, *22*, 238–245.
24. Liu, H.W.; Dong, X.F.; Tong, J.M.; Zhang, Q. A comparative study of growth performance and antioxidant status of rabbits when fed with or without chestnut tannins under high ambient temperature. *Anim. Feed Sci. Technol.* **2011**, *164*, 89–95.
25. Gancarcikova, S.; Nemcova, R.; Popper, M.; Hrckova, G.; Scirankova, L.; Madar, M.; Mudronova, D.; Vilcek, S.; Zitnan, R. The influence of feed-supplementation with probiotic strain *Lactobacillus reuteri* CCM 8617 and alginite on intestinal microenvironment of SPF mice infected with Salmonella Typhimurium CCM 7205. *Probiotics Antimicrob. Proteins* **2019**, *11*, 493–508.
26. Expert Group for Technical Advice on Organic Production (EGTOP). *Final Report on Feed*; EGTOP/1/2011; Directorate-General for Agriculture and Rural Development: Brussels, Belgium, 2011.

27. Dell'Anno, M.; Hejna, M.; Sotira, S.; Caprarulo, V.; Reggi, S.; Pilu, R.; Miragoli, F.; Callegari, M.L.; Panseri, S.; Rossi, L. Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets. *Anim. Feed Sci. Technol.* **2020**, *266*, 114519.
28. Trckova, M.; Lorencova, A.; Babak, V.; Neca, J.; Ciganek, M. The effect of leonardite and lignite on the health of weaned piglets. *Res. Vet. Sci.* **2018**, *119*, 134–142, doi:10.1016/j.rvsc.2018.06.004.
29. Miragoli, F.; Patrone, V.; Prandini, A.; Sigolo, S.; Dell'Anno, M.; Rossi, L.; Senizza, A.; Morelli, L.; Callegari, M.L. Implications of Tributyrin on Gut Microbiota Shifts Related to Performances of Weaning Piglets. *Microorganisms* **2021**, *9*, 584.
30. Sotira, S.; Dell'Anno, M.; Caprarulo, V.; Hejna, M.; Pirrone, F.; Callegari, M.L.; Tucci, T.V.; Rossi, L. Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood Metabolites and Gut Microbiota in Weaned Piglets. *Animals* **2020**, *10*, 726.
31. Murray, R.L.; Zhang, W.; Iwaniuk, M.; Grilli, E.; Stahl, C.H. Dietary tributyrin, an HDAC inhibitor, promotes muscle growth through enhanced terminal differentiation of satellite cells. *Physiol. Rep.* **2018**, *6*, e13706.
32. Zhang, W.-X.; Zhang, Y.; Zhang, X.-W.; Deng, Z.-X.; Liu, J.-X.; He, M.-L.; Wang, H.-F. Effects of Dietary Supplementation with Combination of Tributyrin and Essential Oil on Gut Health and Microbiota of Weaned Piglets. *Animals* **2020**, *10*, 180.
33. Panda, A.; Rao, S.; Raju, M.; Sunder, G.S. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. *Asian Australas. J. Anim. Sci.* **2009**, *22*, 1026–1031.
34. European Commission. *Amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as Regards Mercury, Free Gossypol, Nitrites and Mowrah, Bassia, Madhuca*; Commission Directive EU 6/2010 (Text with EEA relevance); Official Journal of the European Union: Brussels, Belgium, 2010.
35. National Research Council (NRC). *Nutrient Requirements of Swine*, 11th Revised ed.; Animal Nutrition Series; National Research Council: Washington DC, USA; The National Academies Press: Washington, DC, USA, 2012.
36. American Oil Chemistry Society (AOCS). Crude Fiber Analysis in Feeds by Filter Bag Technique. In *Official Methods and Recommended Practices*, 4th ed.; AOCS.: Champaign, IL, USA, 2009.
37. Warke, R.; Kamat, A.; Kamat, M.; Thomas, P. Incidence of pathogenic psychrotrophs in ice creams sold in some retail outlets in Mumbai, India. *Food Control* **2000**, *11*, 77–83, doi:10.1016/S0956-7135(99)00027-4.
38. Henning, C.; Vijayakumar, P.; Adhikari, R.; Jagannathan, B.; Gautam, D.; Muriana, P.M. Isolation and taxonomic identity of bacteriocin-producing lactic acid bacteria from retail foods and animal sources. *Microorganisms* **2015**, *3*, 80–93.
39. Dowell, V.R., Jr.; Hawkins, T.M. *Laboratory Methods in Anaerobic Bacteriology—CDC Laboratory Manual*; Center for Disease Control: Atlanta, GA, USA, 1974.
40. Sales, J.; Janssens, G. Acid-insoluble ash as a marker in digestibility studies: A review. *J. Anim. Feed Sci.* **2003**, *12*, 383–401.
41. Ahmed, S.; Minuti, A.; Bani, P. *In vitro* rumen fermentation characteristics of some naturally occurring and synthetic sugars. *Ital. J. Anim. Sci.* **2013**, *12*, e57.
42. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120.
43. Chong, J.; Liu, P.; Zhou, G.; Xia, J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* **2020**, *15*, 799–821.

44. Dhariwal, A.; Chong, J.; Habib, S.; King, I.L.; Agellon, L.B.; Xia, J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **2017**, *45*, W180–W188.
45. European Commission. *Commission Regulation (EC) No 429/2008, on Detailed Rules for the Implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as Regards the Preparation and the Presentation of Applications and the Assessment and the Authorisation of Feed Additives*; Regulation EC 429/2008 (Text with EEA relevance); Official Journal of the European Union: Brussels, Belgium, 2008.
46. Rychen, G.; Aquilina, G.; Azimonti, G.; Bampidis, V.; Bastos, M.d.L.; Bories, G.; Chesson, A.; Cocconcelli, P.S.; Flachowsky, G. EFSA Panel on Additives and Products or Substances used in Animal Feed: Guidance on the assessment of the efficacy of feed additives. *EFSA J.* **2018**, *16*, e05274.
47. Girard, M.; Thanner, S.; Pradervand, N.; Hu, D.; Ollagnier, C.; Bee, G. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental ETEC F4 model. *PLoS ONE* **2018**, *13*, doi:10.1371/journal.pone.0197878.
48. Hou, Y.; Wang, L.; Yi, D.; Ding, B.; Chen, X.; Wang, Q.; Zhu, H.; Liu, Y.; Yin, Y.; Gong, J.; et al. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. *Br. J. Nutr.* **2014**, *111*, 1748–1758, doi:10.1017/S0007114514000038.
49. Lynegaard, J.; Kjeldsen, N.; Bache, J.; Weber, N.; Hansen, C.; Nielsen, J.; Amdi, C. Low protein diets without medicinal zinc oxide for weaned pigs reduced diarrhoea treatments and average daily gain. *Animal* **2021**, *15*, 100075.
50. Laine, T.M.; Lyytikäinen, T.; Yliaho, M.; Anttila, M. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. *Acta Vet. Scand.* **2008**, *50*, 1–11.
51. Girard, M.; Hu, D.; Pradervand, N.; Neuenschwander, S.; Bee, G. Chestnut extract but not sodium salicylate decreases the severity of diarrhea and enterotoxigenic *Escherichia coli* F4 shedding in artificially infected piglets. *PLoS ONE* **2020**, *15*, e0214267.
52. Girard, M.; Bee, G. Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. *Animal* **2020**, *14*, 95–107.
53. Islam, K.M.S.; Schuhmacher, A.; Gropp, J.M. Humic Acid Substances in Animal Agriculture. *Pak. J. Nutr.* **2005**, *4*, 126–134.
54. Dong, L.; Zhong, X.; He, J.; Zhang, L.; Bai, K.; Xu, W.; Wang, T.; Huang, X. Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets. *Clin. Nutr.* **2016**, *35*, 399–407.
55. Liu, H.; Hu, J.; Mahfuz, S.; Piao, X. Effects of hydrolysable tannins as zinc oxide substitutes on antioxidant status, immune function, intestinal morphology, and digestive enzyme activities in weaned piglets. *Animals* **2020**, *10*, 757.
56. Yu, J.; Song, Y.; Yu, B.; He, J.; Zheng, P.; Mao, X.; Huang, Z.; Luo, Y.; Luo, J.; Yan, H. Tannic acid prevents post-weaning diarrhea by improving intestinal barrier integrity and function in weaned piglets. *J. Anim. Sci. Biotechnol.* **2020**, *11*, 1–11.
57. Wang, C.; Shen, Z.; Cao, S.; Zhang, Q.; Peng, Y.; Hong, Q.; Feng, J.; Hu, C. Effects of tributyrin on growth performance, intestinal microflora and barrier function of weaned pigs. *Anim. Feed Sci. Technol.* **2019**, *258*, 114311.
58. Liu, Y.; Espinosa, C.; Abelilla, J.; Casas, G.; Lagos, L.; Lee, S.; Kwon, W.; Mathai, J.; Navarro, D.; Jaworski, N.; et al. Non-antibiotic feed additives in diets for pigs: A review. *Anim. Nutr.* **2018**, *4*, 113–125, doi:10.1016/j.aninu.2018.01.007.

59. Klem, T.B.; Bleken, E.; Morberg, H.; Thoresen, S.I.; Framstad, T. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. *Vet. Clin. Pathol.* **2010**, *39*, 221–226.
60. Friendship, R.M.; Lumsden, J.H.; McMillan, I.; Wilson, M.R. Hematology and Biochemistry Reference Values for Ontario Swine. *Can. J. Comp. Med.* **1984**, *48*, 390–393.
61. Izsler. Parametri di Chimica Clinica: Valori Osservati in Suini di Diversa Età. Available online: https://www.izsler.it/pls/izs_bs/v3_s2ew_consultazione.mostra_pagina?id_pagina=1494 (accessed on 18 March 2021).
62. Ference, B.A.; Graham, I.; Tokgozoglu, L.; Catapano, A.L. Impact of lipids on cardiovascular health: JACC health promotion series. *J. Am. Coll. Cardiol.* **2018**, *72*, 1141–1156.
63. Buscemi, S.; Corleo, D.; Buscemi, C.; Randazzo, C.; Borzì, A.M.; Barile, A.M.; Rosafio, G.; Ciaccio, M.; Caldarella, R.; Meli, F. Influence of Habitual Dairy Food Intake on LDL Cholesterol in a Population-Based Cohort. *Nutrients* **2021**, *13*, 593.
64. Fechner, A.; Kiehntopf, M.; Jahreis, G. The Formation of Short-Chain Fatty Acids Is Positively Associated with the Blood Lipid–Lowering Effect of Lupin Kernel Fiber in Moderately Hypercholesterolemic Adults. *J. Nutr.* **2014**, *144*, 599–607, doi:10.3945/jn.113.186858.
65. He, J.; Dong, L.; Xu, W.; Bai, K.; Lu, C.; Wu, Y.; Huang, Q.; Zhang, L.; Wang, T. Dietary tributyrin supplementation attenuates insulin resistance and abnormal lipid metabolism in suckling piglets with intrauterine growth retardation. *PLoS ONE* **2015**, *10*, e0136848.
66. Galassi, G.; Mason, F.; Rapetti, L.; Crovetto, G.M.; Spanghero, M. Digestibility and metabolic utilisation of diets containing chestnut tannins and their effects on growth and slaughter traits of heavy pigs. *Ital. J. Anim. Sci.* **2019**, *18*, 746–753.
67. Sahakyan, N.; Bartoszek, A.; Jacob, C.; Petrosyan, M.; Trchounian, A. Bioavailability of Tannins and Other Oligomeric Polyphenols: A Still to Be Studied Phenomenon. *Curr. Pharmacol. Rep.* **2020**, *6*, 131–136.
68. Kim, Y.; Lee, J.H.; Kim, T.H.; Song, M.H.; Yun, W.; Oh, H.J.; Lee, J.S.; Kim, H.B.; Cho, J.H. Effects of low protein diets added with protease on growth performance, nutrient digestibility of weaned piglets and growing-finishing pigs. *J. Anim. Sci. Technol.* **2021**, doi:10.5187/jast.2021.e49.
69. Pieper, R.; Janczyk, P.; Schumann, R.; Souffrant, W. The intestinal microflora of piglets around weaning with emphasis on lactobacilli. *Arch. Zootech.* **2006**, *9*, 28–40.
70. Castillo, M.; Martín-Orúe, S.M.; Manzanilla, E.G.; Badiola, I.; Martín, M.; Gasa, J. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet. Microbiol.* **2006**, *114*, 165–170.
71. Wang, M.; Huang, H.; Hu, Y.; Huang, J.; Yang, H.; Wang, L.; Chen, S.; Chen, C.; He, S. Effects of dietary microencapsulated tannic acid supplementation on the growth performance, intestinal morphology, and intestinal microbiota in weaning piglets. *J. Anim. Sci.* **2020**, *98*, doi:10.1093/jas/skaa112.
72. Maes, D.; Chantziaras, I.; Vallaey, E.; Demeyere, K.; Meyer, E.; Janssens, G.P. Faecal pH throughout the reproductive cycle of sows in commercial pig herds. *J. Anim. Physiol. Anim. Nutr.* **2020**.
73. Dehority, B.A. Microbial ecology of cell wall fermentation. *Forage Cell Wall Struct. Dig.* **1993**, 425–453, doi:10.2134/1993.foragecellwall.c17.
74. Schautteet, K.; Vanrompay, D. *Chlamydiaceae* infections in pig. *Vet. Res.* **2011**, *42*, 1–10.

75. Rogers, D.G.; Andersen, A.A. Intestinal lesions caused by a strain of *Chlamydia suis* in weanling pigs infected at 21 days of age. *J. Vet. Diagn. Investig.* **2000**, *12*, 233–239.
76. Nietfeld, J.C.; Janke, B.H.; Leslie-Steen, P.; Robison, D.J.; Zeman, D.H. Small intestinal *Chlamydia* infection in piglets. *J. Vet. Diagn. Investig.* **1993**, *5*, 114–117.
77. Clavel, T.; Lepage, P.; Charrier, C. The family coriobacteriaceae. *Prokaryotes* **2014**, *11*, 201–238.
78. Gill, S.R.; Pop, M.; Deboy, R.T.; Eckburg, P.B.; Turnbaugh, P.J.; Samuel, B.S.; Gordon, J.I.; Relman, D.A.; Fraser-Liggett, C.M.; Nelson, K.E. Metagenomic analysis of the human distal gut microbiome. *Science* **2006**, *312*, 1355–1359.
79. Ran, S.; Mu, C.; Zhu, W. Diversity and community pattern of sulfate-reducing bacteria in piglet gut. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1–11.
80. Unno, T.; Choi, J.-H.; Hur, H.-G.; Sadowsky, M.J.; Ahn, Y.-T.; Huh, C.-S.; Kim, G.-B.; Cha, C.-J. Changes in human gut microbiota influenced by probiotic fermented milk ingestion. *J. Dairy Sci.* **2015**, *98*, 3568–3576.
81. Yang, Q.; Huang, X.; Zhao, S.; Sun, W.; Yan, Z.; Wang, P.; Li, S.; Huang, W.; Zhang, S.; Liu, L. Structure and function of the fecal microbiota in diarrheic neonatal piglets. *Front. Microbiol.* **2017**, *8*, 502.
82. Amat, S.; Lantz, H.; Munyaka, P.M.; Willing, B.P. *Prevotella* in Pigs: The Positive and Negative Associations with Production and Health. *Microorganisms* **2020**, *8*, 1584.
83. Wang, X.; Tsai, T.; Deng, F.; Wei, X.; Chai, J.; Knapp, J.; Apple, J.; Maxwell, C.V.; Lee, J.A.; Li, Y. Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome* **2019**, *7*, 1–18.
84. Sun, J.; Du, L.; Li, X.; Zhong, H.; Ding, Y.; Liu, Z.; Ge, L. Identification of the core bacteria in rectums of diarrheic and non-diarrheic piglets. *Sci. Rep.* **2019**, *9*, 1–10.

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Article

Lactobacillus plantarum and *Lactobacillus reuteri* as Functional Feed Additives to Prevent Diarrhoea in Weaned Piglets

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Brief Introduction to the Study:

The following manuscript represent a novel contribution to the field of animal nutrition. The research conducted in this study was part of a European project funded by Lombardy Region and European Rural Development Fund (Foodtech project "innovative products in the zootechnical field") and evaluated the use of two strains of probiotic bacteria not routinary used in pig nutrition. *L. plantarum* and *L. reuteri* isolated from pigs were evaluated for probiotic characteristics *in vitro* in order to assess their survival during the digestive process and important technological characteristics for their suitability to feed formulations were investigated. The results showed that *L. plantarum* and *L. reuteri* were resistant to the simulated digestive process *in vitro,* and reduced diarrhoea occurrence *in vivo*. In particular, *L. plantarum* registered the highest ability to prevent gastrointestinal disorders, also showing an increase of blood globulin concentration, which are often linked to immunity. The results of the study underlined the positive impact of *L. plantarum* and *L. reuteri* as innovative additives for pig nutrition in order to improve pig health, animal welfare and reduce the use of antibiotic treatments.

4.6 *Lactobacillus plantarum* and *Lactobacillus reuteri* as Functional Feed Additives to Prevent Diarrhoea in Weaned Piglets

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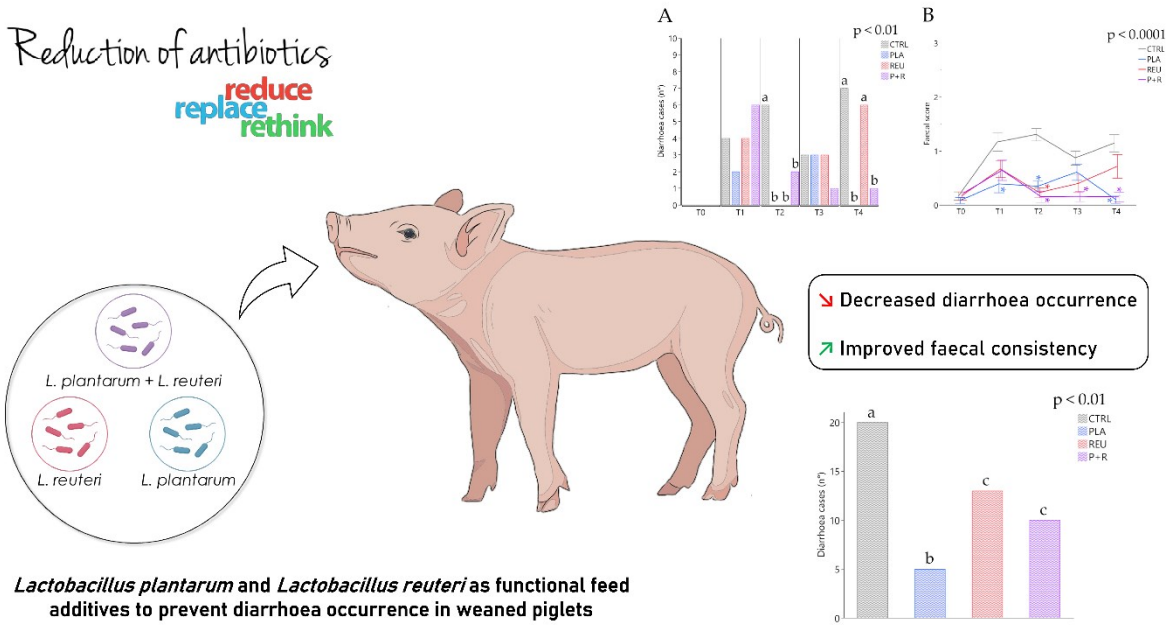
Simple Summary: Antimicrobial resistance is an increasing global concern. Effective alternatives that could replace and reduce antimicrobial treatments in farming have therefore become crucial for animal, human and environmental health. In swine farming, weaning is a stressful phase where piglets can develop multifactorial enteric disorders that require antibiotic treatments. Functional nutrition could thus represent a valuable alternative to reduce and tackle antibiotic resistance. This study assesses the effects of *Lactobacillus plantarum* and *Lactobacillus reuteri* on in-feed supplementation in weaned piglets. After weaning, piglets were allotted to four experimental groups fed a basal diet (CTRL) and a basal diet supplemented with 2×10^8 CFU/g of *L. plantarum* (PLA), *L. reuteri* and a combination of the two strains (P+R) for 28 days. Zootechnical performance and diarrhoea occurrence were recorded. Microbiological and serum metabolism analyses of faeces and blood samples were performed. Supplemented groups with lactobacilli showed a lower occurrence of diarrhoea and improved faecal consistency compared to the control. The PLA group registered the lowest diarrhoea frequency during the 28-day experimental period. The results suggest that dietary administration of *L. plantarum* and *L. reuteri* could prevent the occurrence of diarrhoea in weaned piglets.

Graphical abstract:



Abstract: The effects of *Lactobacillus plantarum* and *Lactobacillus reuteri* and their combination were assessed in weaned piglets. Three hundred and fifty weaned piglets (Landrace × Large White), balanced in terms of weight and sex, were randomly allotted to four experimental groups (25 pens, 14 piglets/pen). Piglets were fed a basal control diet (CTRL, six pens) and a treatment diet supplemented with 2×10^8 CFU/g of *L. plantarum* (PLA, 6 pens), 2×10^8 CFU/g *L. reuteri* (REU, six pens) and the combination of both bacterial strains (1×10^8 CFU/g of *L. plantarum* combined with 1×10^8 CFU/g of *L. reuteri*, P+R, 7 pens) for 28 days. Body weight and feed intake were recorded weekly. Diarrhoea occurrence was assessed weekly by the faecal score (0–3; considering diarrhoea ≥ 2). At 0 and 28 days, faecal samples were obtained from four piglets per pen for microbiological analyses and serum samples were collected from two piglets per pen for serum metabolic profiling. Treatments significantly reduced diarrhoea occurrence and decreased the average faecal score (0.94 ± 0.08 CTRL, 0.31 ± 0.08 PLA, 0.45 ± 0.08 REU, 0.27 ± 0.08 P+R; $p < 0.05$). The PLA group registered the lowest number of diarrhoea cases compared to other groups (20 cases CTRL, 5 cases PLA, 8 cases REU, 10 cases P+R; $p < 0.01$). After 28 days, the globulin serum level increased in PLA compared to the other groups (24.91 ± 1.09 g/L CTRL, 28.89 ± 1.03 g/L PLA, 25.91 ± 1.03 g/L REU, 25.31 ± 1.03 g/L P+R; $p < 0.05$). *L. plantarum* and *L. reuteri* could thus be considered as interesting functional additives to prevent diarrhoea occurrence in weaned piglets.

Keywords: *Lactobacillus plantarum*; *Lactobacillus reuteri*; probiotics; lactobacilli; functional nutrition; diarrhoea prevention; intestinal health; weaned pig

1. Introduction

In livestock farming, effective alternatives to antibiotics that are able to promote health and prevent pathologies are urgently required to tackle antibiotic resistance [1–3], and replacing and reducing antibiotic treatments is one of the main targets of European policies [4]. This became even more important after the removal from the market of zinc oxide (ZnO) as a veterinary therapeutic treatment [5,6]. This decision was taken due to the observed increase in heavy metal environmental pollution and scientific evidence showing that ZnO co-selects antibiotic-resistant bacteria [7,8]. ZnO has been used widely after the ban on antibiotics as a growth promoter over the last decade [6,9–11]. Alternatives to ZnO and antibiotics are thus required particularly during the weaning phase due to the high incidence of enteric disorders and multifactorial diseases such as post-weaning diarrhoea (PWD) [12,13]. The gastroenteric tract (GIT) is a complex environment where the mucosal chemical barrier, immune system, microbiota and epithelium all impact intestinal health [14,15]. Preserving intestinal health decreases the incidence of pathologies, optimises digestive processes and promotes animal performance. There is increased awareness regarding

the role of diet, not only as a physiological requirement, but also in the enhancement of animal and human health and in the prevention of specific pathologies [16]. The modulation of intestinal microbiota by dietary approaches, such as the use of feed additives, is one of the most promising strategies to reduce the risk of pathologies in food-producing animals [17,18].

Probiotics are functional feed additives defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [19]. Their potential mechanisms of action affect the intestinal microbial ecology through the manipulation of microbiota that lower the luminal pH, the competitive inhibition of pathogen strains, the production of bacteriocins with antimicrobial proprieties and the stimulation of the host immune system [20]. Probiotic supplementation in animal diets helps prevent or treat a variety of intestinal disorders, although their mechanisms of actions are not completely known [21]. Lactic acid bacteria include over two-hundred species and subspecies of which *Lactobacillus* sp., *Lactococcus* sp., *Spreptococcus* sp. and *Enterococcus* sp. are used as probiotics for monogastric animals [22].

Lactobacillus plantarum is included in the European register of feed additives [8] as a preservative (1; a), silage additive (1; k), microorganism (1; k) and gut flora stabilizer for chickens (4; b). In several *in vitro* and *in vivo* studies, some strains of *L. plantarum* demonstrated a protective activity against epithelial intestinal barrier impairment, restoring the function of thigh junctions and reducing paracellular permeability [23,24]. In addition, *L. plantarum* CGMCC 1258 supplemented at 5×10^{10} CFU/kg showed its positive effect in weaned piglets challenged with *Escherichia coli* K88, inhibiting diarrhoea and improving zootechnical performance [25]. In parallel, *Lactobacillus reuteri* was included in the EU feed additive register as a microorganism (1; k) until its withdrawal in 2012 [8] due to a lack of the required documentation. This microorganism is not seen as being dangerous and no issues related to its safety were mentioned in the EU commission decision [26], since it is included in the Qualified Presumption of Safety (QPS) list of the European Union [27].

Lactobacillus reuteri I5007 has shown a potential to improve thigh junction expression in newborn piglets and has been found to have protective effects after lipo-polysaccharide (LPS)-induced stress *in vitro* [21]. *L. reuteri* strains TMW1.656 and LTH5794 produce reuteran which can decrease the adhesive capacity of ETEC *E. coli* [28]. However, several studies have shown the positive impact of various *L. plantarum* and *L. reuteri* strains on improving piglet performance, diarrhoea prevention, stress alleviation, immunity and microbiota modulation [29].

Since few papers have assessed the effects of *L. plantarum* and *L. reuteri* strains and their synergy through a wide range of bacterial combination and supplementation levels, more studies are required to clarify the functional proprieties and the optimal

inclusion level of these two bacterial strains on diarrhoea prevention in weaned piglets. In addition, probiotics may interact with the host metabolism [30] through their hypocholesterolemic and liver protection effects [31,32]. Furthermore, the bacterial combination does not always result in a synergistic effect, also showing possible competition among probiotic strains [33]. The aim of the study was to evaluate *L. plantarum*, *L. reuteri* and whether their combined supplementation reflects synergistic or antagonistic effects on diarrhoea prevention, metabolic status and performance in weaned piglets.

2. Materials and Methods

2.1. Species-Specific PCR

Single colonies of *L. plantarum* and *L. reuteri,* isolated from swine, obtained from the Biotecnologie BT (Perugia, Italy) strain collection were cultured in De Man, Rogosa and Sharpe (MRS) medium for 24 h in anaerobiosis conditions at 37 °C. Bacterial strains were diluted in 20 µL of lysis solution (microLYSIS® solution, Clent Life Science, Stourbridge, England) and thermically lysed following the manufacturer's instructions. After lysis, in order to confirm bacterial species, 2 µL of extracted DNA was used for a PCR reaction through species-specific primers, following the protocol previously described by Torriani et al. [34] for *L. plantarum* and Song et al. [35] for *L. reuteri*. PCR reaction was performed with 17 µL of PCR master mix (Client Life Science, Stourbridge, England) and 0.5 µL (0.25 µM) of specific primers. *L. plantarum* ATCC®14917™ and *L. reuteri* DSM 20016 DNA were included as positive controls.

2.2. Minimal Inhibitory Concentration (MIC)

In order to assess the possible presence of antibiotic-resistant genes in *L. plantarum* and *L. reuteri* isolated strains, a minimal inhibitory concentration test was performed. MICs were assessed following ISO 10932 IDF 223 guidelines, adopting VetMIC Lact-1 (version 1) and VetMIC Lact-2 (version 2) (National Veterinary Institute, SVA) (Annex I and II). *L. plantarum* ATCC®14917™ was included as a positive control. *L. reuteri* and *L. plantarum* were tested for 16 antibiotic molecules (gentamicin, kanamycin, streptomycin, neomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, penicillin, vancomycin, quinupristin/dalfopristin, linezolid, trimethoprim, ciprofloxacin and rifampicin).

2.3. Gastric Acid and Simulated In Vitro Digestion Resistance

Bacterial cultures of *L. reuteri* and *L. plantarum* were diluted in MRS broth in order to obtain an optical density of 0.1 measured at 600 nm (V-630 UV-vis, Jasco

Deutschland GmbH, Germany). For the gastric acid tolerance test, both bacterial strains were incubated at different pH levels (2, 3, 4, 5, 7 and the control, i.e., medium in which the pH had not been changed), obtained by adding HCl (1 M) monitoring with a pH meter. To perform this assay, bacterial cultures were incubated for 1 h at 30 °C. Bacterial cultures were then diluted and plated on MRS agar using the overlay method [36]. Plates were incubated at 30 °C and the colonies were counted after 48 h. Strain tolerances to *in vitro*-simulated gastrointestinal tract (GIT) conditions were evaluated according to Charteris et al. [37] and Jensen et al. [38], with minor adaptations. Three independent assays were performed for each strain. The MRS broth with lactobacillus inoculum were incubated at 37 °C for 24 h in anaerobic conditions. To simulate the oral phase, a 10-mL bacterial culture aliquot was added to 10 mL of a sterile electrolyte solution (0.22 g/L $CaCl_2$, 16.2 g/L NaCl, 2.2 g/L KCl and 1.2 g/L $NaHCO_3$) containing 2.0 g/L pepsin (Sigma-Aldrich Co., Saint Louis, MO, USA) and the first sampling was performed. A gastric resistance assay was performed by adjusting the pH to 3.0 by the addition of HCl (1 M) to activate pepsinogen. The sampling was performed after 90 min of incubation at 37 °C under stirring. A total of 2 mL was then sampled in two tubes for each strain and the cell pellets were obtained by centrifugation at 12,000 rpm for 5 min at 4 °C. In order to simulate duodenal shock phase, the bacterial pellet of one tube for each strain was resuspended in 2 mL of sterile saline solution (16.30 g K_2HPO_4, 0.9 g KH_2PO_4) supplemented with 0.25 g of porcine bile extract (Oxgall, Merck, Darmstadt, Germany) and subsequently sampled after 10 min of incubation at 37 °C. To evaluate lactobacilli resistance to intestinal conditions, the bacterial pellet of the remaining tube of each strain was resuspended in 2 mL of sterile saline solution containing 0.075 g of porcine bile extract and 0.025 g of porcine pancreatin (Sigma-Aldrich Co., Saint Louis, MO, USA). The last sampling was performed after the incubation for 240 min at 37 °C. Bacterial viability was assessed by plate counting on MRS agar for each sampling point using the overlay method [36]. Plates were then incubated at 30 °C for 48 h, and visible colonies were enumerated.

2.4. Small-Scale Fermentation and Freeze-Drying Resistance

In order to optimise biomass production conditions for experimental trial dietary inclusion, a small-scale fermentation was adopted. A 3 L bioreactor was employed to produce bacterial biomass inoculating fresh *L. reuteri* and *L. plantarum* cultures in 2.5 L of MRS (pH 5.2) supplemented with 2% of glucose maintained at 37 °C, stirred at 10 rpm, to harvest bacteria after 18 and 24 h. Bacterial biomasses were weighted after centrifugation at 4 °C, 4800 rpm, for 25 min. Viability was assessed by resuspending bacterial biomass and performing plate counting after 48 h of incubation under anaerobic conditions at 37 °C. Bacterial samples stored at −80 °C were freeze-dried and

samples were heated for 1440 min with 0.2 mbar of pressure for the condenser. The biomass obtained and the vitality of lactobacilli strains were measured by weighting and plate counting, respectively.

2.5. Bacterial Fermentation for Experimental Trial Batch Production

Large-scale fermentations were adopted following the previously described conditions. A total of 3 L of fresh bacterial cultures were inoculated to 30 L of MRS (pH 5.2) supplemented with 1% of saccharose, maintained at 35 °C, and stirred at 10 rpm for 24 h. Biomass was harvested through centrifugation at 4200 rpm, 4 °C for 45 min and cryopreservation solution (43 g/L Na citrate, 28.6 g/L glucose, 28.6 g/L saccharose, 28.6 g/L milk powder and 28.6 g/L ascorbic acid; 1:2, *w*/*v*) was added before freee-drying.

2.6. Experimental Design, Animal Housing and Dietary Treatments

The experimental trial was performed in accordance with European regulations [39] and approved by the Animal Welfare Organisation of University of Milan (OPBA authorisation n° 09/2020). The *in vivo* trial was performed on a commercial farm free from pathologies included in the ex-list A of World Organization of Animal Health (OIE): atrophic rhinitis, Aujeszky disease, porcine reproductive respiratory syndrome, salmonellosis and transmissible gastroenteritis. Three-hundred and fifty piglets (Landrace × Large White) weaned at 28 ± 2 days and homogeneous in terms of sex (50% male and 50% female) and weight (7.48 ± 1.07 kg) were identified by individual ear tags and randomly divided into four experimental groups. Animals were allotted in 25 different pens (14 piglets/pen) in standardised environmental conditions (27 °C, 60% relative humidity) for 28 days. After three days of an adaptation period when the animals were fed the same basal diet in order to enable them to overcome the typical post weaning fasting, piglets were assigned to four experimental groups and were fed ad libitum: the control group (CTRL: 84 piglets, 6 pens, 7.46 ± 0.13 kg) the basal diet; the *L. plantarum* treated group (PLA: 84 piglets, 6 pens, 7.49 ± 0.12 kg) basal diet supplemented with 2×10^8 CFU/g of *Lactobacillus plantarum*; the *L. reuteri* treated group (REU: 84 piglets, 6 pens, 7.62 ± 0.12 kg) basal diet plus 2×10^8 CFU/g of *L. reuteri,* and the *L. plantarum* and *L. reuteri* combination group (P+R: 98 piglets, 7 pens, 7.36 ± 0.11 kg) fed basal diet plus 1×10^8 CFU/g of both bacterial strains. Treatments were balanced for each group. The P+R group was characterised by one additional pen in order to include the entire trial room in the experimental design. All the diets were isoproteic and isoenergetic (Table 1) balanced using Plurimix System®software (Fabermatica, Cremona, Italy) in line with nutritional requirements for post-weaned piglets [40], and were provided by Ferraroni S.p.A. (Cremona, Italy). Considering the

small amount of freeze-dried lactobacilli powder included, the bacterial strains were premixed with wheat flour to ensure a homogeneous dispersion before being added to the horizontal mixer. For the whole diet, 2% of the wheat meal was substituted with 2% of the experimental mix (wheat flour + bacterial strain in order to reach a concentration in the final preparation of 2×10^8 CFU/g).

Experimental diets were analysed in duplicate for lactobacilli viability by plate counting and principal nutrient content [41]: dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash concentrations. DM was obtained by drying samples in pre-weighed aluminium jars through a forced air oven at 65 °C. CP was determined by the Kjeldahl method. EE was assessed by performing ether extraction in a Soxtec. CF was determined by the filtering bag method. Ash content was measured after incinerating samples in a muffle furnace at 550 °C. The fatty acid profile of the experimental diets was analysed starting from a total lipid extraction and the fatty acid methyl esters were prepared according to Christie and Han [42]. The fatty acid analysis was carried out using gas chromatography (TRACE GC Ultra, Thermo Fisher Scientific, Rodano, Italy) fitted with an automatic sampler (AI 1300, Thermo Fisher Scientific) and flame ionization detector (FID). An RT-2560 fused silica capillary column (100 m × 0.25 mm × 0.25 μm film thickness; Restek, Milan, Italy) was used with a programmed temperature from 80 °C to 180 °C at 3 °C/min, then from 180 °C to 250 °C at 2.5 °C/min, which was then held for 10 min. The carrier gas was helium at 1.0 mL/min with an inlet pressure of 16.9 psi. A quantitative procedure was used where 1 mL of internal standard (1 mg/mL 23:0 methyl ester; N-23-M; Nu-Chek Prep Inc., Elysian, MN, USA) was added prior to methylation. The fatty acid methyl ester (FAME) contents were quantified by weight as a percentage of the total FAMEs. All analyses were performed in duplicate.

Table 1. Diet composition and principal chemical characteristics of experimental trial (% as fed basis) divided by control (CTRL, fed basal diet) and treatment groups (TRT, fed basal diet supplemented with 2×10^8 CFU/g of *Lactobacillus plantarum*; 2×10^8 CFU/g of *Lactobacillus reuteri* and 1×10^8 CFU/g of both bacterial strains; PLA, REU and P+R, respectively).

Ingredients, % as Fed Basis	CTRL	TRT
Barley, meal	26.84	26.84
Wheat, meal	12.45	10.45
Corn, flakes	11.63	11.63
Corn, meal	10.00	10.00
Barley, flakes	7.50	7.50
Soy protein concentrates	5.00	5.00
Biscuits, meal	4.00	4.00
Soybean, meal	4.00	4.00
Dextrose monohydrate	3.50	3.50
Sweet milk whey	3.20	3.20
Herring, meal	2.00	2.00

Plasma, meal	2.00	2.00
Organic acids [1]	1.70	1.70
Coconut oil	1.00	1.00
Soy oil	1.00	1.00
Arbocel [2]	0.70	0.70
Dicalcium phosphate	0.60	0.60
L-Lysine	0.60	0.60
Benzoic acid	0.50	0.50
Vitamin and mineral premix [3]	0.50	0.50
DL-Methionine	0.39	0.39
L-Threonine	0.35	0.35
Sodium Chloride	0.27	0.27
L-Valine (96.5%)	0.12	0.12
Enzymes [4]	0.10	0.10
L-Tryptophan	0.05	0.05
Experimental mix [5]	-	2.00
Calculated Chemical Composition [6]		
Crude protein (%)	17.00	17.00
Fat (%)	4.20	4.20
Crude fibre (%)	2.90	2.90
Ashes (%)	5.20	5.20
DE [7] (Mc/Kg)	3.92	3.83

[1] Citric acid, fumaric acid, orthophosphoric acid, sorbic acid, calcium formate. [2] Crude fibre concentrate (Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany).[3] Additives per Kg: Vitamins, pro-vitamins and substances with similar effect. Retinyl Acetate 15,000 IU, Vitamin D3-Cholecalciferol 2000 IU, Vitamin E 120 mg, Vitamin B1 2.0 mg, Vitamin B2 4.8 mg, Vitamin B6 3.4 mg, Calcium D-pantothenate 15.0 mg, Vitamin B12 0.030 mg, Vitamin K3 1.9 mg, Biotin 0.19 mg, Niacinamide 30.0 mg, Folic Acid 0.96 mg, Vitamin C 144 mg, Choline chloride 288 mg, Betaine hydrochloride 1000 mg, Compounds of trace elements Iron sulphate 115 mg, Manganese Oxide 48.0 mg, Zinc Oxide 96.1 mg, Copper Oxide 130 mg, Anhydrous Calcium Iodate 0.96 mg, Sodium Selenite 0.34 mg. [4] 6-phytase, endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase.[5] Experimental mix was composed of wheat flour 00, and the respective bacterial strain according to dietary treatments: *L. plantarum* (PLA), *L. reuteri* (REU), *L. plantarum* and *L. reuteri* combination (P+R) in order to reach a final concentration of 2 × 108 CFU/g in the complete diet. [6] Calculation performed with Purimix System® software (Fabermatica, Cremona, Italy).[7] DE: digestible energy content estimated from NRC (2012).

2.7. Animal Performance, Diarrhoea Occurrence and Biological Sample Collection

Body weight (BW) was recorded individually at day 0 (T0), day 7 (T1), day 14 (T2), day 21 (T3) and day 28 (T4). Feed intake was recorded weekly by measuring the feed refused for each pen, considering the pen as the experimental unit. Other performance parameters: average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated. Four piglets per pen were randomly selected for faecal sample collection and microbiological analysis (24 piglets CTRL, 24 piglets PLA, 24 piglets REU, 28 piglets P+R; balanced per weight and sex) for the entire experimental period.

Diarrhoea occurrence was recorded weekly by evaluating the faecal consistency which was given a faecal score: a four-level scale (0 = dried consistency, 1 = soft

consistency, 2 = mild diarrhoea, 3 = severe diarrhoea). Faecal colour was evaluated through a three-level colour scale: 1 = yellowish, 2 = greenish, 3 = brown; considering ≥ 2 as a normal score [12].

Blood samples were obtained from the jugular vein at T0 and T4 through vacuum tubes from two randomly selected piglets per pen, balanced in terms of weight and sex, maintained over time for the entire experimental period.

2.8. Microbiological and pH Evaluation of Faecal Samples

Faecal samples were analysed for the total countable bacteria, lactic acid bacteria and coliform bacteria through three different types of culture media: Plate Count Agar (PCA), De Man, Rogosa and Sharpe Agar (MRS) and Violet Red Bile Broth Agar (VRBA), respectively. One gram of faecal sample was diluted and homogenised with 10 mL of sterile 0.9% NaCl solution and centrifugated (3000 rpm, 10 min) to collect the supernatants. Samples were then serially diluted tenfold, and microorganisms were enumerated by plate counting after 24 h of incubation at 37 °C. The lactic acid/coliform bacteria ratio was calculated based on plate counting data from MRS and VRBA agar. The results were expressed as $\log_{10}$ of colony-forming units per gram of faeces ($\log_{10}$ CFU/g). Fresh faecal samples of pH of T4, diluted in 10 mL of 0.9% NaCl solution and subsequently centrifugated, were measured on the supernatant through a pH meter.

2.9. Serum Metabolites

Serum samples were obtained by centrifugation (3000 rpm, 15 min) and analysed for the concentration of: total protein (g/L), albumin (g/L), globulin (g/L), albumin/globulin (A/G ratio), alanine aminotransferase (ALT-GPT; IU/L), glucose (mmol/L), urea (mmol/L), creatinine (μmol/L), total bilirubin (μmol/L), total cholesterol (mmol/L), triglycerides (mmol/L), high-density lipoprotein (HDL; mmol/L), low-density lipoprotein (LDL; mmol/L), phosphorus (mmol/L) and magnesium (mmol/L) levels with a multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA) at 37 °C. Serum concentration of interleukins 3, 6 and 10 were also quantified immunoenzymatically using enzyme-linked immunosorbent assay (ELISA) kits specific for swine species according to the manufacturer's instructions (Bioassay Technology Laboratory, Shanghai, China), and concentrations were calculated by fitting the relative standard curves with CurveExpert 1.4 software.

2.10. Statistical Analysis

The results were analysed using a repeated-measures ANOVA using JMP 14 Pro® (SAS Inst. Inc., Cary, NC, USA). Zootechnical performance, faecal score data and faecal

bacterial counts were evaluated using a full factorial model (Treatment: Trt, Time: Time, Interaction: Trt × Time). Data related to acid resistance, *in vitro* simulated digestion, blood metabolism and faecal pH at T4 were assessed through analysis of variance (ANOVA). Diarrhoea incidence was obtained by converting the faecal score data into a dichotomous variable (presence or absence) in order to evaluate observed frequencies through the Pearson's Chi-Squared test. Multiple comparisons among groups were evaluated by performing Tukey's Honest Significance Difference test (Tukey's HSD). The results were presented as least square means ± standard errors (SE). The means were considered different when $p \leq 0.05$ and statistically tendent for $0.09 \leq p < 0.05$.

3. Results

3.1. Species-Specific PCR

PCR reaction confirmed the expected fragment of 318 bp for *L. plantarum* and 303 bp for *L. reuteri* (Figure S1).

3.2. Minimal Inhibitory Concentrations

The results of the MIC concentrations tested revealed a bacterial susceptibility to a wide range of antibiotics (Table S1).

3.3. Acid and Simulated In Vitro Digestion Resistance

Bacterial strains exposed to a different pH range showed a statistically significant drop in viability at pH 2, with *L. plantarum* and *L. reuteri* registering a bacterial count of 8.09 ± 0.11 and 9.00 ± 0.02 $\log_{10}$ CFU/mL, respectively ($p < 0.0001$), compared to their relative controls at pH 7 (9.60 ± 0.08 and 10.79 ± 0.02 $\log_{10}$ CFU/mL, respectively) (Figure 1). Regarding the simulated gastrointestinal digestion, both bacterial strains exhibited an optimal capacity to survive with each tested condition, including gastric juice, bile shock and intestinal juice, without registering any significant decrease in viability compared to their relative initial microbial charge (Figure 2).

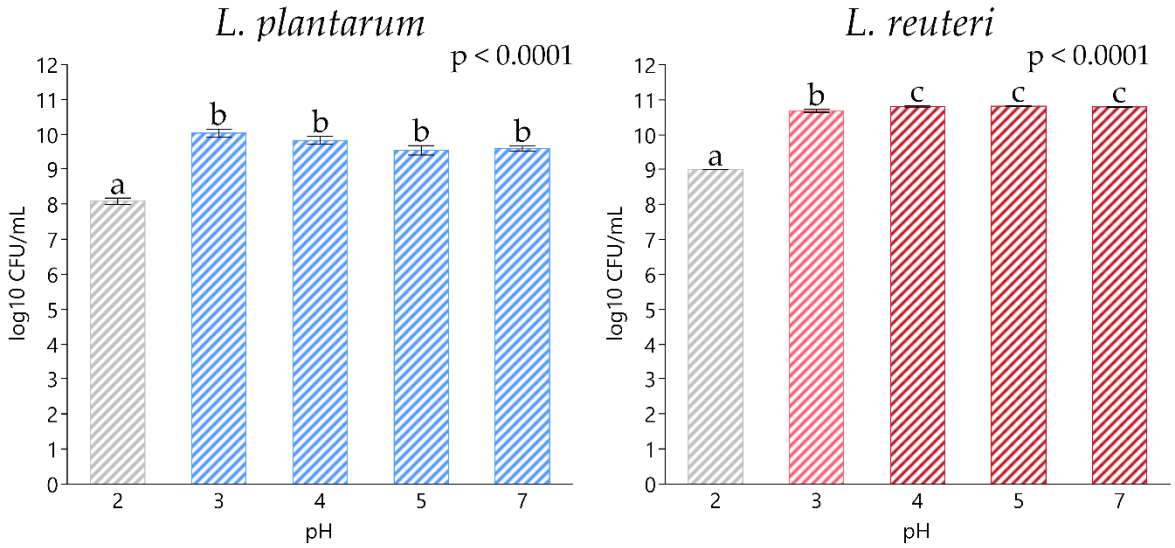


Figure 1. Acid resistance assay from pH 2 to 7 for *L. plantarum* and *L. reuteri*. Data are expressed as least square means (LSMEANS) and standard errors (SE). [a–b–c] Means with different superscript letters indicate statistically significant differences ($p < 0.05$).

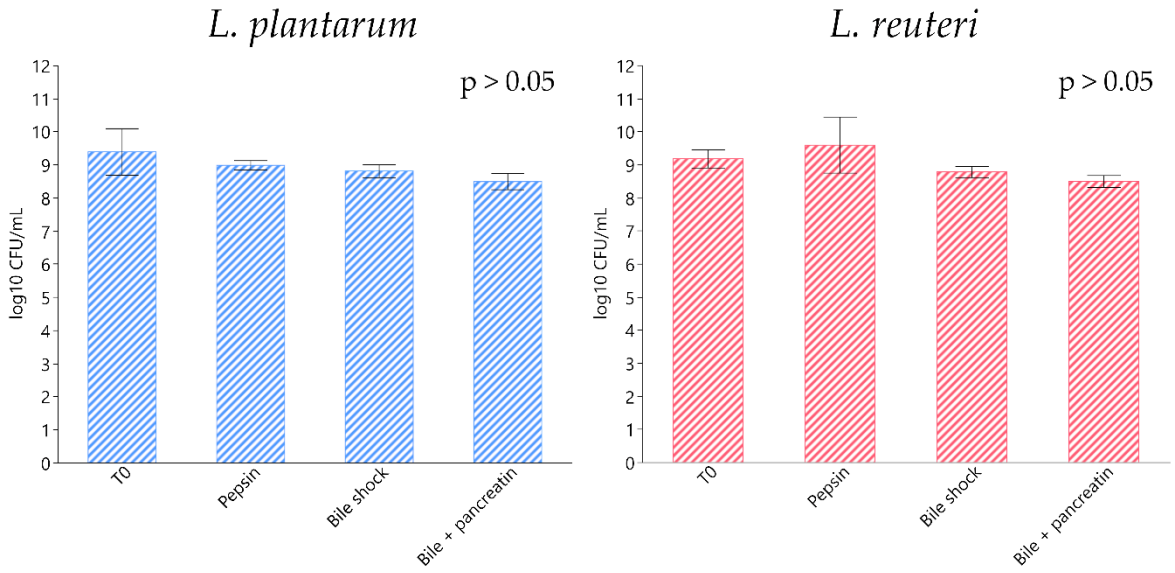


Figure 2. Simulated *in vitro* gastrointestinal digestion resistance of *L. plantarum* and *L. reuteri.* Data are expressed as least square means (LSMEANS) and standard errors (SE). T0 corresponds to the initial microbial charge measured before gastrointestinal environment simulation.

3.4. Small-Scale Fermentation and Freeze-Drying Resistance

The results of small-scale fermentations showed similar biomass gain and CFU/g viability for both strains considering 18 and 24 h of fermentation time and registering 2.25×10^{11} CFU/g biomass for *L. plantarum* and 1.72×10^{11} CFU/g biomass for *L. reuteri* after 24 h of fermentation (Table S2). Freeze-drying led to a loss in viability of about 1 log considering the initial lactobacilli count (Table S3).

3.5. Bacterial Fermentation for Experimental Trial Batch Production

Large-scale fermentation performed with 30 L of bacterial culture produced 206.15 and 376.43 g of *L. plantarum* and *L. reuteri* biomass, respectively (Table S4). The freeze-dried bacteria were then used to prepare the experimental diets for a final concentration in feed of 2×10^8 CFU/g.

3.6. Evaluation of Experimental Diets

Experimental diet evaluation of lactobacilli viability and principal nutrient content revealed a bacterial viability loss of 10% and nutrient concentrations in line with NRC [40] guidelines, thus fulfilling the nutritional requirements of weaned piglets. The inclusion of bacterial strains did not influence the nutrient profile of treatment groups (Table 2).

Table 2. Chemical composition of experimental diets divided by control (CTRL) and treatment groups (PLA, REU and P+R).

Analyte	**CTRL**	**PLA**	**REU**	**P+R**
DM	90.89	91.14	91.14	90.78
CP	16.34	17.01	16.38	16.64
EE	3.98	3.78	3.74	3.80
CF	3.60	3.65	3.34	3.40
Ashes	4.59	4.49	4.54	4.25
FA Composition (% Total FAMEs)	**CTRL**		**TRT**	
Caproic acid, C6:0	0.04		0.04	
Caprylic acid, C8:0	1.00		1.10	
Capric acid, C10:0	1.12		1.21	
Undecanoic acid, C11:0	0.00		0.00	
Lauric acid, C12:0	10.85		11.69	
Tridecanoic acid, C13:0	0.01		0.01	
Myristic acid, C14:0	5.23		5.45	
Mysticoleic acid, C14:1	0.01		0.01	
Pentadecanoic acid, C 15:0	0.05		0.06	
cis-10 Heptadecenoic acid, C 17:0	0.00		0.00	
Stearic acid, C 18:0	15.17		14.95	
Elaidic acid, C 18:1 n9t	0.23		0.22	
Oleic acid, C 18:1 n9c	0.09		0.08	
Linolelaidic acid, C18:2 n6t	0.00		0.00	
Linoleic acid, C 18:2 n6c	4.34		4.07	
γ-Linolenic acid, C 18:3 n6	0.05		0.04	
α-Linolenic acid, C18:3 n3	22.71		22.50	
Arachidic acid, C 20:0	0.00		0.00	
Cis-11 Eicosenoic acid, C20:1	34.42		33.92	
Cis-11,14 Eicosenoic acid, C20:2	0.04		0.04	
Cis-8,11,14 Eicosatrienoic acid, C20:3 n6	2.65		2.74	
Cis-11,14,17 Eicosatrienoic acid, C20:3 n3	0.31		0.29	
Arachidonic acid, C20:4 n6	0.43		0.40	
Cis-5,8,11,14,17 Eicosapentaenoic acid, C20:5 n3	0.05		0.04	
Heneicosanoic acid, C21:0	0.00		0.00	

Behenic acid, C22:0	0.01	0.01
Erucic acid, C22:1 n9	0.02	0.02
Cis-13,16 Docosadienoic acid, C22:2	0.26	0.25
Cis-4,7,10,13,16,19 Docosahexaenoic acid, C22:6 n3	0.02	0.02
Lignoceric acid; C24:0	0.25	0.23
Nervonic acid, C24:1	0.04	0.04
SFA	38.64	39.36
MUFA	23.52	23.25
PUFA	37.83	37.39

DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fibre; FA: fatty acids; FAMEs: fatty acid methyl esters; SFA: saturated fatty acids, MUFA monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. All values are expressed as percentage as fed basis (%). CTRL: control group; PLA: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum*; REU: treatment group supplemented with 2×10^8 CFU/g of *L. reuteri;* P+R: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum* and *L. reuteri* (1:1 *w*/*w*); TRT: treatment group supplemented with 2×10^8 CFU/g of lactobacilli.

3.7. Zootechnical Performance

The results of individual BW recorded weekly showed no significant differences throughout the experimental period (Figure 3). The average BW of CTRL and PLA groups revealed a statistically significant tendency compared to P+R considering the entire experimental period (10.45 ± 0.19; 10.42 ± 0.17; 9.84 ± 0.16 kg, respectively; $p < 0.09$). In addition, the effect of treatments on ADG for the entire experimental period was significantly different for CTRL, REU and P+R groups, which showed a reduced average gain for treated groups with *L. reuteri* (CTRL: 260 ± 9, REU: 220 ± 8, P+R: 229 ± 7 g/day; $p < 0.05$). The ADFI of the supplemented groups decreased during the second week (7–14 days) of the study (CTRL: 490 ± 24, PLA: 281 ± 24; REU: 334 ± 24, P+R: 318 ± 22 g/day; $p < 0.01$). The FCR parameter highlighted an increased ratio in P+R group compared to CTRL, PLA and REU during the first week (0–7 days; CTRL: 2.89 ± 0.24; PLA: 2.67 ± 0.24; REU: 2.67 ± 0.24; P+R: 4.32 ± 0.23; $p < 0.01$).

3.8. Diarrhoea Occurrence

Considering the entire experimental period, diarrhoea observed frequencies differed significantly among treatments ($p < 0.01$). The highest number of cases of diarrhoea (20 cases) was found in the CTRL group, while 13 and 10 cases were recorded in the REU and P+R groups, respectively. The lowest number of diarrhoea cases (five cases) was recorded in the PLA group (Figure 4). Data on diarrhoea incidence considering each timepoint showed a statistically significant increase in CTRL compared to the treated groups at T2 (CTRL: 6 cases, 25.00%; PLA: 0 cases, 0.00%; REU 0 cases, 0.00%; P+R: 2 cases; 7.14%; $p < 0.01$) (Figure 5A). At the last sampling point (T4), diarrhoea occurrence was significantly lower in the PLA and P+R groups (CTRL: 7 cases; 29.17%; PLA: 0 cases, 0.00%; REU: 6 cases, 25.00%; P+R: 1 case; 3.57%; $p < 0.01$). Average faecal scores of representative subgroups of evaluated piglets

revealed a higher score for the CTRL group compared with PLA at T1 (CTRL: 1.17 ± 0.13; PLA: 0.40 ± 0.13; $p < 0.01$) (Figure 5B). The average faecal score of CTRL after 14 days (T2) increased significantly compared with the treatment groups (CTRL: 1.31 ± 0.13; PLA: 0.34 ± 0.13; REU: 0.24 ± 0.13; P+R: 0.16 ± 0.12; $p < 0.0001$). At 21 days (T3), P+R highlighted a lower score compared to the CTRL group (CTRL: 0.89 ± 0.13; P+R: 0.18 ± 0.13; $p < 0.05$). PLA and P+R groups showed a significant decrease in average faecal score at the end of the trial compared to the CTRL group (CTRL: 1.16 ± 0.14; PLA: 0.13 ± 0.14; P+R: 0.17 ± 0.12; $p < 0.0001$).

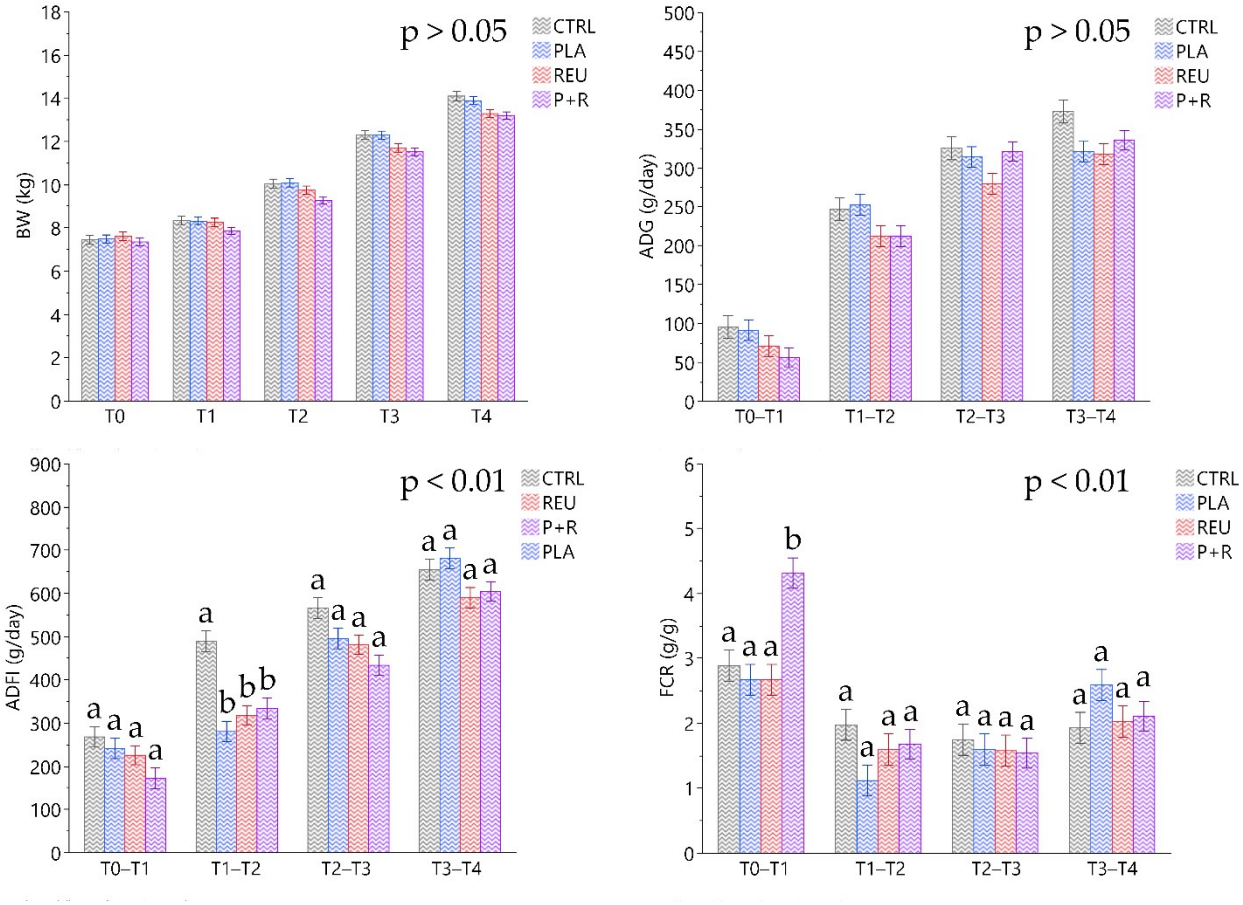


Figure 3. Zootechnical performance of control (CTRL) and treatment groups (PLA, REU and P+R) measured over 28 days of experimental trial. Data are expressed as least square means (LSMEANS) and standard errors of the means (SE); [a–b] Means with different superscripts are significantly different among treatments ($p < 0.01$); Presented p-values indicate statistically significances of pairwise comparisons; BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FRC: feed conversion ratio; CTRL: control group; PLA: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum*; REU: treatment group supplemented with 2×10^8 CFU/g of *L. reuteri*; P+R: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum* and *L. reuteri* (1:1 *w*/*w*).

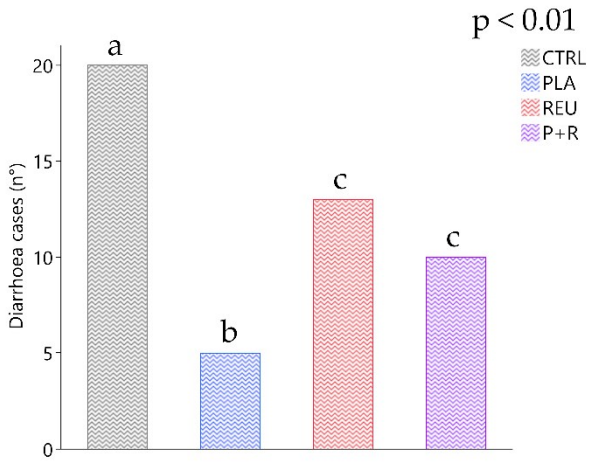


Figure 4. Total diarrhoea cases recorded during the 28-day trial for the control (CTRL) and treatment groups (PLA, REU and P+R). Data are expressed as the sum of recorded cases of diarrhoea, considering a faecal score ≥ 2 diarrhoea; [a–b-c] Means with different superscripts are significantly different among treatments ($p < 0.01$). CTRL: control group; PLA: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum*; REU: treatment group supplemented with 2×10^8 CFU/g of *L. reuteri;* P+R: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum* and *L. reuteri* (1:1, *w*/*w*).

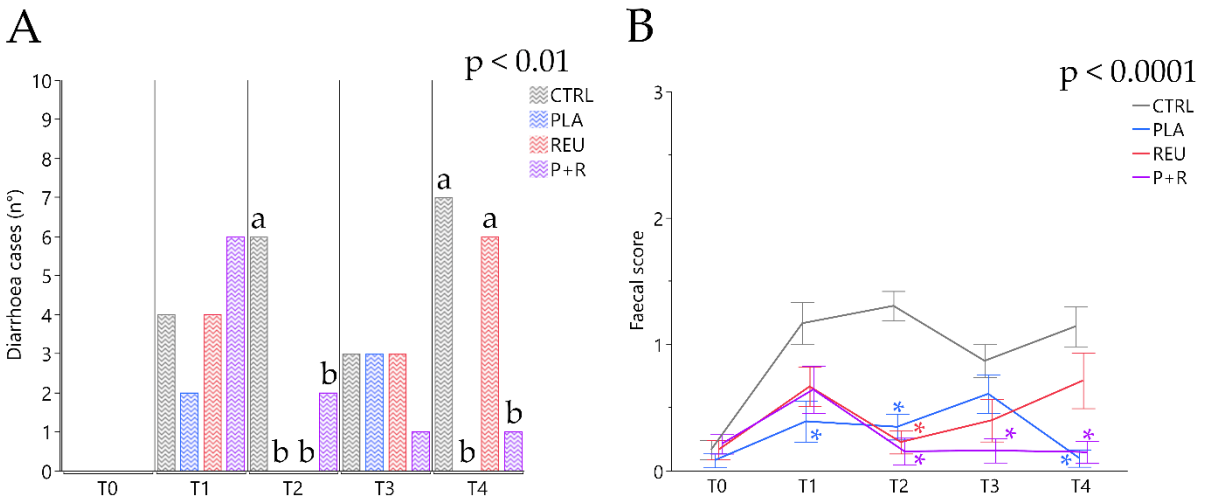


Figure 5. Number of diarrhoea cases recorded (**A**) and average faecal scores (**B**) during the 28-day trial for the control (CTRL) and treatment groups (PLA, REU and P+R). (**A**) Data are expressed as the sum of the recorded cases of diarrhoea, considering faecal score ≥ 2 diarrhoea; [a–b] Means with different superscripts are significantly different among treatments ($p < 0.01$). (**B**) Data are expressed as least square means (LSMEANS) and standard errors (SE); * Means with asterisks are significantly different from the control group (CTRL, $p < 0.0001$). CTRL: control group; PLA: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum*; REU: treatment group supplemented with 2×10^8 CFU/g of *L. reuteri*; P+R: treatment group supplemented with 2×10^8 CFU/g of *L. plantarum* and *L. reuteri* (1:1, *w*/*w*).

3.9. Microbiological Analysis and Faecal pH

Bacterial plate count results revealed no statistically significant difference among experimental groups at day 0 (T0) and after 28 days of the trial (T4) (Figure 6). However, a statistical tendency was observed for the lactic acid/coliform bacteria ratio at T4 comparing the CTRL and PLA groups (1.08 ± 0.10 and 1.54 ± 0.08 CFU/g, respectively; $p < 0.09$). Faecal pH measured at T4 revealed comparable averages among

CTRL and treated groups (CTRL: 7.00 ± 0.07; PLA: 7.02 ± 0.08; REU: 7.24 ± 0.10; P+R: 7.09 ± 0.08).

3.10. Serum Metabolism

The results of serum metabolites showed no statistically significant differences over time for all experimental groups at T0 (Table S5). After 28 days, the PLA group showed a statistically significant increase in globulin content compared to the other groups (Table 3; $p < 0.05$). Consequently, the albumin/globulin ratio of the PLA group was lower than the other experimental groups ($p < 0.05$). Alanine aminotransferase (ALT) decreased significantly in the PLA and REU groups compared to the other groups ($p < 0.01$). The phosphorous concentration was higher in the P+R compared to PLA and REU groups ($p < 0.05$). The PLA group showed a decreased magnesium content in serum compared to the other groups ($p < 0.05$). Total cholesterol was lower in PLA and REU compared to the other experimental treatments ($p < 0.05$). In fact, high density lipoproteins were lower in PLA and REU compared to CTRL and P+R treatments ($p < 0.01$).

Table 3. Serum metabolites concentration at 28 days (T4) of *in vivo* trial, for the control (CTRL) and treatments groups (PLA, REU and P+R).

Serum Metabolite	CTRL	PLA	REU	P+R	*p*-Value
Total protein content, g/L	53.26 ± 1.23	54.85 ± 1.15	51.84 ± 1.15	52.09 ± 1.15	0.2576
Albumin, g/L	28.35 ± 0.74	26.00 ± 0.70	25.94 ± 0.70	26.77 ± 0.70	0.0916
Globulin, g/L	24.91 ± 1.09 ^{a}	28.89 ± 1.03 ^{b}	25.91 ± 1.03 ^{a}	25.31 ± 1.03 ^{a}	0.0455
Albumin/Globulin (A/G)	1.16 ± 0.05 ^{a}	0.92 ± 0.05 ^{b}	1.06 ± 0.05 ^{ab}	1.01 ± 0.05 ^{ab}	0.0287
Urea, mmol/L	1.06 ± 0.21	1.42 ± 0.20	0.96 ± 0.20	0.89 ± 0.20	0.2452
Alanine aminotransferase (ALT-GPT), IU/L	50.00 ± 2.89 ^{a}	38.22 ± 2.73 ^{b}	35.78 ± 2.73 ^{b}	46.00 ± 2.73 ^{ab}	0.0034
Total bilirubin, μmol/L	1.84 ± 0.13	1.42 ± 0.12	1.45 ± 0.12	1.58 ± 0.12	0.1021
Glucose, mmol/L	6.36 ± 0.47	6.31 ± 0.44	5.36 ± 0.44	6.41 ± 0.44	0.2926
Phosphorus, mmol/L	3.19 ± 0.09 ^{ab}	2.87 ± 0.08 ^{a}	2.98 ± 0.08 ^{a}	3.30 ± 0.08 ^{b}	0.0038
Magnesium, mmol/L	0.92 ± 0.04 ^{a}	0.77 ± 0.11 ^{b}	0.79 ± 0.12 ^{ab}	0.85 ± 0.13 ^{ab}	0.0196
Creatinine, μmol/L	70.75 ± 3.51	77.33 ± 3.31	76.00 ± 3.31	81.10 ± 3.31	0.2175
Total cholesterol, mmol/L	2.70 ± 0.14 ^{a}	2.20 ± 0.13 ^{b}	2.27 ± 0.13 ^{b}	2.68 ± 0.13 ^{a}	0.0195
High density lipoprotein (HDL), mmol/L	1.08 ± 0.06 ^{a}	0.77 ± 0.06 ^{b}	0.81 ± 0.06 ^{b}	1.04 ± 0.06 ^{a}	0.0011
Low density lipoprotein (LDL), mmol/L	1.50 ± 0.09	1.26 ± 0.09	1.31 ± 0.09	1.52 ± 0.09	0.1173
Triglycerides, mmol/L	0.58 ± 0.08	0.83 ± 0.07	0.70 ± 007	0.60 ± 0.07	0.0764
Interleukin 3, pg/L	17.80 ± 1.98	14.78 ± 2.17	17.28 ± 2.17	17.90 ± 2.17	0.7098
Interleukin 6, pg/L	166.47 ± 45.87	152.65 ± 45.87	155.06 ± 45.87	166.48 ± 45.87	0.9941
Interleukin 10, pg/L	10.67 ± 2.13	8.91 ± 2.13	8.49 ± 2.13	10.80 ± 2.13	0.8158

Data are expressed as least square means (LSMEANS) ± standard errors (SE). $^{a–b}$ Means with different superscripts are significantly different among treatments ($p < 0.05$). CTRL: control group; PLA: treatment group supplemented with 2×10^{8} CFU/g of *L. plantarum*; REU: treatment group supplemented

with 2 × 10^8 CFU/g of *L. reuteri;* P+R: treatment group supplemented with 2 × 10^8 CFU/g of *L. plantarum* and *L. reuteri* (1:1, *w*/*w*).

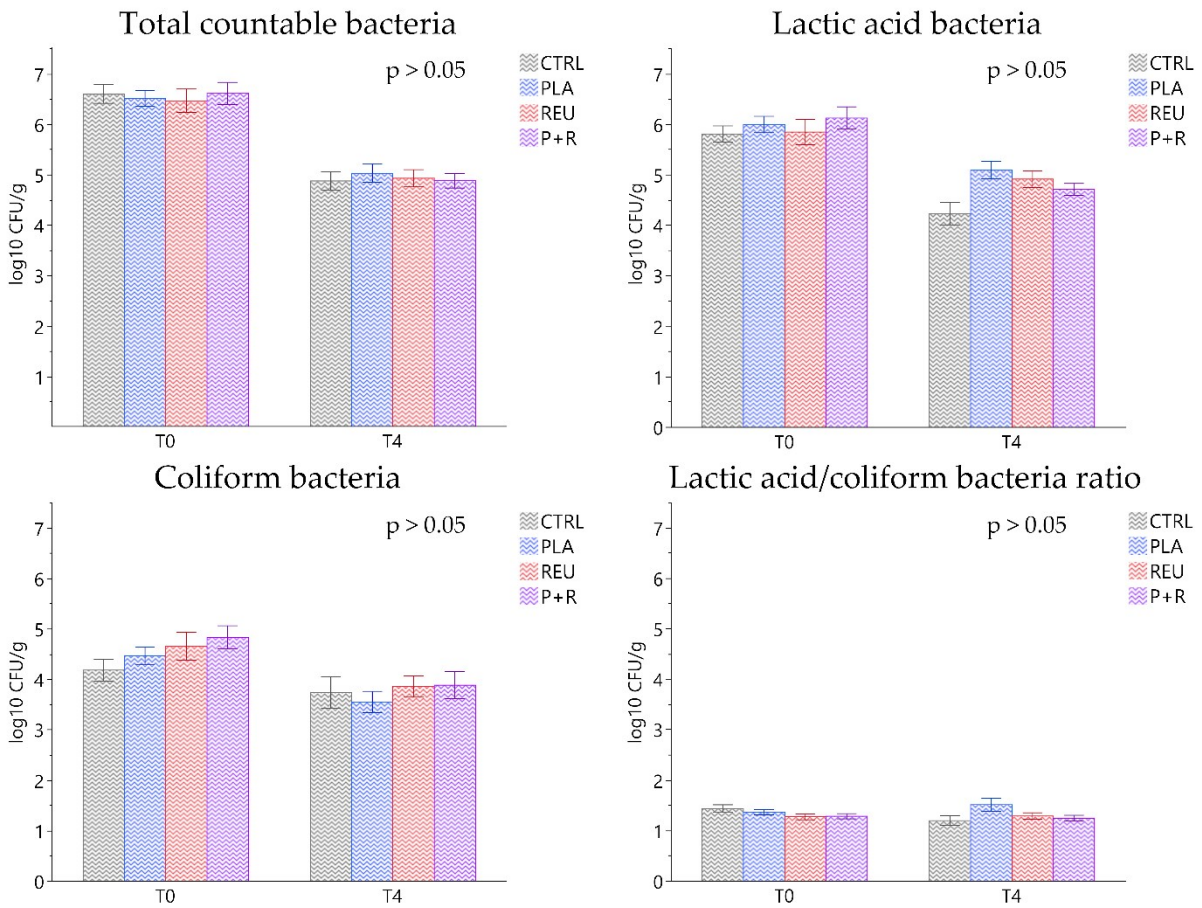


Figure 6. Faecal colonies of the principal bacterial groups (total countable bacteria, lactic acid bacteria, coliform bacteria and lactic acid/coliform ratio) for the control (CTRL) and treatment groups (PLA, REU and P+R) measured at the beginning (T0) and after 28 days of the trial (T4). Data are expressed as least square means (LSMEANS) and standard errors (SE). CTRL: control group; PLA: treatment group supplemented with 2 × 10^8 CFU/g of *L. plantarum*; REU: treatment group supplemented with 2 × 10^8 CFU/g of *L. reuteri;* P+R: treatment group supplemented with 2 × 10^8 CFU/g of *L. plantarum* and *L. reuteri* (1:1, *w*/*w*).

4. Discussion

Weaning is a critical phase characterised by a high incidence of gastrointestinal disorders. Probiotics may support intestinal health during this particular phase. This study focused on the effects of the dietary supplementation of *L. plantarum* and *L. reuteri* and their combination on the performance, metabolic status and gut health in weaned piglets. Lactic acid bacteria need to be ingested when administered as probiotics. They therefore need to reach the intestinal environment in a viable state in order to exert their wide range of positive activities. Probiotic bacteria are thus required to pass through the gastric environment where the pH reaches 2.5 [43].

The results related to acid tolerance showed that both *L. plantarum* and *L. reuteri* tolerate pH levels above 2 without losing significant viability. In line with our study, Yun et al. [44] demonstrated that *L. plantarum* and *L. reuteri* were able to resist pH levels from 4 to 9, meanwhile at pH 2, both strains showed a similar survival reduction (20%) after 6 h of incubation. Lukacova et al. [45] reported that more than 90% of *L. plantarum* strains need to survive at pH 3 in order to act as probiotics.

In vitro simulated gastrointestinal tract transit tolerance is an important assay to evaluate the properties of probiotics. *L. plantarum* and *L. reuteri* strains showed an efficient ability to survive under each tested condition. In fact, *L. plantarum* and *L. reuteri* tolerate the gastric and small intestinal environment depending on the strain tested [38,46,47]. Bove et al. [48] evaluated the survival ability of *L. plantarum* WCFS1 in an oro-gastric-intestinal tract model, highlighting that this particular strain survives the entire digestion process. Our results suggest that *L. plantarum* and *L. reuteri* strains could be provided in feed and reach the intestinal environment without a significant viability loss, also without the need for other protection forms (e.g., microencapsulation).

The nutrient profile of experimental diets assessed by our chemical analyses was in line with post weaning piglet requirements following NRC guidelines [40]. Our experimental design considered a three-day adaptation period in order to enable piglets to adapt to the new environment and to be accustomed to feeding only on a solid diet, thus overcoming post-weaning fasting. Zootechnical performance is key to farm profitability and also an indirect index of animal health. Body weight showed a constant increase over the 28 days of our trial without significant differences among groups. Although the daily gain calculated for the 28 days was lower for REU and P+R than for CTRL, there were no differences considering all experimental groups for each timepoint (T1-T2-T3-T4). This thus highlighted that this slight reduction did not significantly impact the final body weight, ADG, ADFI and FCR of the animals. Other studies have shown a significant increase in body weight and average daily gain by including *L. plantarum* in the pig diet [25]. *L plantarum* supplemented at 10^9 CFU/d showed an improved weight gain when administered for 60 days [49]. Bentancur et al. [50] orally administered 10^9 CFU of *L. plantarum* CAM-6 from 21 to 49 days of age and found an increased daily gain and no differences in feed intake. In addition, *L. reuteri* strains supplementation have been shown to improve animal performance in weaned piglets [29,51]. Wang et al. [52] observed an increased feed intake and average daily gain with high doses of *L. reuteri* X-1 (10^{11} CFU/kg). Although several studies have shown boosting activity related to animal performance, the results are not directly comparable due to the different bacterial genotypes tested, animal ages, particular probiotic combinations, different in-feed inclusion levels or disparate

supplementation methods. Furthermore, in line with EFSA guidelines [53], zootechnical performance effects should be better clarified with a long-term study supplementing probiotics at higher dosages (1×10^9 CFU/g).

Gastrointestinal disorders are a major problem in swine farming during the weaning phase, when diarrhoea is one of the most evident dysbiosis signs and one of the principal reasons for prescribing antibiotics. On the other hand, eubiosis represents a healthy gut that is achieved through a positive interaction between the host, microorganisms and the environment. Our results revealed a lower diarrhoea occurrence in the lactobacilli-supplemented groups. The lowest diarrhoea cases were recorded in the *L. plantarum*-supplemented group (PLA). Over time, the lactobacilli-supplemented groups showed a lower faecal score, indicating an improved faecal consistency. The positive effect on diarrhoea was observed by administering different *L. plantarum* and *L. reuteri* strains to piglets individually. The prevention effects of *L. plantarum* on ETEC K88 have been observed *in vitro* and *in vivo* in a pig model through the stimulation of claudin-1, zonula occludens (ZO-1) and occludin expression, preventing epithelial barrier disruption [25,54]. Our results are in line with other studies where *L. reuteri* decreased diarrhoea incidence in piglets. In fact, *L. reuteri* supplemented at 2.4×10^5 CFU/g as a lactobacilli preparation complex decreased diarrhoea incidence by over 60% [55]. The in-feed supplementation of *L. plantarum* and *L. reuteri* confirms their positive contribution to eubiosis in the intestinal environment. In addition, these results suggest that single strains or a combination of these lactic acid bacteria could help prevent diarrhoea.

The bacterial plate count after weaning (T0) highlighted a high prevalence of lactic acid bacteria, and a reduction in this class was observed after 28 days (T4). In general, during the neonatal phase, lactobacilli and lactic acid bacteria are more common in piglets due to the consumption of maternal milk. They decrease in the post weaning phase frequently due to solid diet feeding [56]. During the trial, statistical differences in faecal viable bacterial counts were not detected among treatments and the control group. However, De Angelis et al. [57] found high viable lactobacilli in piglet faeces when 1×10^{10} CFU/pig *L. plantarum* 4.1 and *L. reuteri* 3S7 were administered for 15 days. The lactic acid/coliform bacteria ratio can be considered as a practical index for efficacy tests of feed additives, aimed at promoting the immune defence. Higher values of the lactobacilli:coliform ratio are normally associated with increased resistance to intestinal disorders [58]. Even only a statistical tendency of PLA group was registered compared to CTRL for this index at T4. The whole lactobacilli supplemented groups showed a lactic acid/coliform bacteria ratio above 1.3. On the other hand, CTRL showed a similar prevalence of lactic acid and coliform bacteria, suggesting that lactic acid was more predominant than coliform bacteria in supplemented groups.

The serum metabolic profile was useful in evaluating animals' health and nutritional status, in order to clarify the possible interaction between bacteria and the host metabolism. Our results revealed that individual lactobacilli and their combined supplementation was safe without impairing animal metabolism, since all the values are in the normal range for pigs. The metabolic parameters showed higher levels of globulin which directly reduced the A/G ratio in PLA compared to the CTRL group. Globulins are mainly represented by immunoglobulins and are an important marker of immune system activity. Our results are in line with Dong et al. [59] who found a significant increase in globulin, with a simultaneous decrease in the A/G ratio after five weeks of *L. plantarum* GF103 supplementation. In addition, an increased concentration of IgA was observed by combining *L. plantarum* GF103 and *Bacillus subtilis* B27 [59]. The administration of microencapsulated *L. plantarum* and fructooligosaccharide blend has been found to increase plasma IgA and IgG concentrations in pigs [60]. Naqid et al. [61] observed that *L. plantarum* B2984 and lactulose dietary supplemented enhanced IgG production in response to *Salmonella typhimurium* infection in pigs. ALT can be exploited as a serum marker of liver damage, whose increase is related to cell membrane damage. Alanine aminotransferase is specific for liver tissues and is more effective in assessing a decrease in cell liver damage [62]. Although our results are in line with normal range for pigs [63–66], PLA and REU groups showed a significant reduction in serum ALT at 28 days suggesting a possible protective effect of *L. plantarum* and *L. reuteri* on liver cells. Fang et al. [32] showed that *L. plantarum* CMU995 supplementation decreased the ALT levels inhibiting alcohol-induced hepatitis. In line with our results, many probiotic species (*L. acidophilus*, *L. bulgaricus*, *Bifidobacterium lactis*, *Streptococcus termophylus*) demonstrated a protective effect on liver [67]. Phosphorous and magnesium are fundamental coenzymes and regulate many biochemical reactions in mammals. The P+R group had a higher P content after 28 days of supplementation with the lactobacilli combination. The magnesium serum concentration was lower in the PLA group than in other treatments. The interaction of dietary nutrients and the activity of microbiota are directly involved in mineral absorption [68]. P+R and PLA groups serum mineral concentrations suggest that *L. plantarum* administration could modulate mineral utilisation. Total cholesterol serum level is an index of the lipometabolic status, which includes the free and bounded forms of HDL [69]. The PLA and REU groups showed a significant reduction in total cholesterol, mainly due to the registered decrease of HDL concentration. *L. plantarum* and *L. reuteri* have been reported as positively contributing to cardiovascular diseases [70,71]. Certain probiotic strains could enhance faecal excretion of bile acids and resulting in a decrease of serum cholesterol concentration [72]. In line with this, *L. plantarum* 9-41-A significantly

decreased hepatic cholesterol and TG levels when administered to rats fed a high-cholesterol diet [73]. Our results are likely due to the ability of the lactobacilli strain to modulate lipid metabolism, thereby preventing hypercholesterolemia.

5. Conclusions

Dietary supplementation of 2 × 10^8 CFU/g of *L. plantarum* and *L. reuteri* significantly reduced diarrhoea occurrence registering and had the lowest faecal score in our trial. *L. plantarum* had the lowest diarrhoea frequency compared to the other bacterial strains and their combinations. Lactobacilli supplementation did not influence animal performance, total faecal bacteria, faecal lactobacilli and coliform. Dietary lactobacilli inclusion did not reveal metabolic status alteration ascribable to a pathological status. In particular, *L. plantarum* significantly raised the globulin levels, suggesting a possible stimulation of the immune system. In conclusion, we believe that *L. plantarum* and *L. reuteri* are promising functional feed additives for preventing pig diarrhoea. More studies are required to enrich knowledge of these bacterial strains, to assess their effect for longer experimental periods, and to optimise their possible delivery systems.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11061766/s1, Figure S1: Agarose gel electrophoresis of *L. plantarum* PCR reaction products, Figure S2: Agarose gel electrophoresis of *L. reuteri* PCR reaction products, Table S1: MIC concentrations (μg/mL) obtained for *L. plantarum* and *L. reuteri* strains and cut-off values proposed from EFSA guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, Table S2: Obtained biomass from small-scale fermentations and viability of *L. plantarum* and *L. reuteri*, Table S3: Obtained biomass and relative viability of freeze-dried *L. reuteri* and *L. plantarum*, Table S4: Bacterial fermentation for experimental trial batch production, Table S5: Serum metabolites concentration at 0 days (T0) of *in vivo* trial, for the control (CTRL) and treatments groups (PLA, REU and P+R).

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funding acquisition: L.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and the experimental trial was approved by the Animal Welfare Organization of University of Milan (OPBA authorization n_ 09/2020).

Data Availability Statement: The data presented in this study are available within the article and supplementary materials.

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References

1. Arsène, M.M.; Davares, A.K.; Andreevna, S.L.; Vladimirovich, E.A.; Carime, B.Z.; Marouf, R.; Khelifi, I. The use of probiotics in animal feeding for safe production and as potential alternatives to antibiotics. *Vet. World* **2021**, *14*, 319.
2. Ng, W.J.; Shit, C.-S.; Ee, K.Y.; Chai, T.T. Plant Natural Products for Mitigation of Antibiotic Resistance. In *Sustainable Agriculture Reviews 49*; Springer: Berlin/Heidelberg, Germany, 2021; pp. 57–91.
3. Tang, K.L.; Caffrey, N.P.; Nóbrega, D.B.; Cork, S.C.; Ronksley, P.E.; Barkema, H.W.; Polachek, A.J.; Ganshorn, H.; Sharma, N.; Kellner, J.D. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: A systematic review and meta-analysis. *Lancet Planet. Health* **2017**, *1*, e316–e327.
4. Cormican, M.; Hopkins, S.; Jarlier, V.; Reilly, J.; Simonsen, G.; Strauss, R.; Vandenberg, O.; Zabicka, D.; Zarb, P.; Catchpole, M.; et al. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and

antimicrobial consumption in humans and food-producing animals. *EFSA J.* **2017**, *15*, doi:10.2903/j.efsa.2017.5017.
5. European Medicines Agency. *EMA/394961/2017. European Medicines Agency, Questions and Answers on Veterinary Medicinal Products Containing Zinc Oxide to be Administered Orally to Food-Producing Species Outcome of a Referral Procedure under Article 35 of Directive 2001/82/EC (EMEA/V/A/118)*;EMA: London, 2017.
6. Hejna, M.; Onelli, E.; Moscatelli, A.; Bellotto, M.; Cristiani, C.; Stroppa, N.; Rossi, L. Heavy-Metal Phytoremediation from Livestock Wastewater and Exploitation of Exhausted Biomass. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2239.
7. Bonetti, A.; Tugnoli, B.; Piva, A.; Grilli, E. Towards Zero Zinc Oxide: Feeding Strategies to Manage Post-Weaning Diarrhea in Piglets. *Animals* **2021**, *11*, 642.
8. EU Commission. *Regulation EC 1831/2003. of the European Parliament and of the Council, of 22 September 2003 on Additives for Use in Animal Nutrition (Text with EEA Relevance)*; EU Commission: Belgium, 2003.
9. Hejna, M.; Gottardo, D.; Baldi, A.; Dell'Orto, V.; Cheli, F.; Zaninelli, M.; Rossi, L. Nutritional ecology of heavy metals. *Animal* **2018**, *12*, 2156–2170.
10. Hejna, M.; Moscatelli, A.; Onelli, E.; Baldi, A.; Pilu, S.; Rossi, L. Evaluation of concentration of heavy metals in animal rearing system. *Ital. J. Anim. Sci.* **2019**, *18*, 1372–1384, doi:10.1080/1828051X.2019.1642806.
11. Hejna, M.; Moscatelli, A.; Stroppa, N.; Onelli, E.; Pilu, S.; Baldi, A.; Rossi, L. Bioaccumulation of heavy metals from wastewater through a Typha latifolia and Thelypteris palustris phytoremediation system. *Chemosphere* **2020**, *241*, doi:10.1016/j.chemosphere.2019.125018.
12. Rossi, L.; Dell'Orto, V.; Vagni, S.; Sala, V.; Reggi, S.; Baldi, A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic Escherichia coli strain in piglets. *Vet. Res. Commun.* **2014**, *38*, 39–49, doi:10.1007/s11259-013-9583-9.
13. Lu, C.W.; Wang, S.E.; Wu, W.J.; Su, L.Y.; Wang, C.H.; Wang, P.H.; Wu, C.H. Alternative antibiotic feed additives alleviate pneumonia with inhibiting ACE-2 expression in the respiratory system of piglets. *Food Sci. Nutr.* **2021**, *9*, 1112–1120.
14. Chelakkot, C.; Ghim, J.; Ryu, S.H. Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp. Mol. Med.* **2018**, *50*, 1–9.
15. Sarkar, A.; Yoo, J.Y.; Valeria Ozorio Dutra, S.; Morgan, K.H.; Groer, M. The association between early-life gut microbiota and long-term health and diseases. *J. Clin. Med.* **2021**, *10*, 459.
16. Domínguez Díaz, L.; Fernández-Ruiz, V.; Cámara, M. The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 1738–1746.
17. Lallès, J.P.; Montoya, C.A. Dietary alternatives to in-feed antibiotics, gut barrier function and inflammation in piglets post-weaning: Where are we now? *Anim. Feed Sci. Technol.* **2021**, 114836.
18. Tomičić, Z.; Čabarkapa, I.; Čolović, R.; Đuragić, O.; Tomičić, R. Salmonella in the feed industry: Problems and potential solutions. *J. Agron.* **2018**, *22*, 2019.
19. Hori, T.; Matsuda, K.; Oishi, K. Probiotics: A Dietary Factor to Modulate the Gut Microbiome, Host Immune System, and Gut–Brain Interaction. *Microorganisms* **2020**, *8*, 1401.

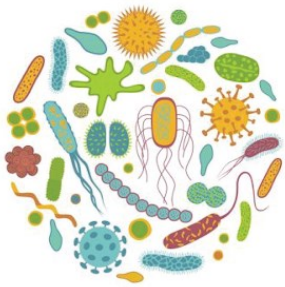
20. Guevarra, R.B.; Lee, J.H.; Lee, S.H.; Seok, M.J.; Kim, D.W.; Kang, B.N.; Johnson, T.J.; Isaacson, R.E.; Kim, H.B. Piglet gut microbial shifts early in life: Causes and effects. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1–10.
21. Yang, F.; Wang, A.; Zeng, X.; Hou, C.; Liu, H.; Qiao, S. Lactobacillus reuteri I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. *BMC Microbiol.* **2015**, *15*, 1–11.
22. Śliżewska, K.; Chlebicz-Wójcik, A.; Nowak, A. Probiotic Properties of New Lactobacillus Strains Intended to Be Used as Feed Additives for Monogastric Animals. *Probiotics Antimicrob. Proteins* **2021**, *13*, 146–162.
23. Lee, C.S.; Kim, S.H. Anti-inflammatory and anti-osteoporotic potential of lactobacillus plantarum A41 and L. fermentum SRK414 as probiotics. *Probiotics Antimicrob. Proteins* **2020**, *12*, 623–634.
24. Gao, Y.; Liu, Y.; Ma, F.; Sun, M.; Song, Y.; Xu, D.; Mu, G.; Tuo, Y. Lactobacillus plantarum Y44 alleviates oxidative stress by regulating gut microbiota and colonic barrier function in Balb/C mice with subcutaneous D-galactose injection. *Food Funct.* **2021**, *12*, 373–386.
25. Yang, K.; Jiang, Z.; Zheng, C.; Wang, L.; Yang, X. Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. *J. Anim. Sci.* **2014**, *92*, 1496–1503.
26. EU Commission. *Regulation EU 451/2012. COMMISSION IMPLEMENTING REGULATION (EU) No 451/2012 on the Withdrawal from the Market of Certain Feed Additives Belonging to the Functional Group of Silage Additives (Text with EEA Relevance)*; EU Commission: Belgium, 2012.
27. EFSA BIOHAZ Panel; Koutsoumanis, K.; Allende, A.; Alvarez-Ordonez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Hilbert, F.; et al. The list of QPS status recommended biological agents for safety risk assessments carried out by EFSA. *EFSA J.* **2021**, doi:10.5281/zenodo.4428353.
28. Chen, X.Y.; Woodward, A.; Zijlstra, R.T.; Gänzle, M.G. Exopolysaccharides synthesized by Lactobacillus reuteri protect against enterotoxigenic Escherichia coli in piglets. *Appl. Environ. Microbiol.* **2014**, *80*, 5752–5760.
29. Hou, C.; Zeng, X.; Yang, F.; Liu, H.; Qiao, S. Study and use of the probiotic Lactobacillus reuteri in pigs: A review. *J. Anim. Sci. Biotechnol.* **2015**, *6*, 14.
30. Ghini, V.; Tenori, L.; Pane, M.; Amoruso, A.; Marroncini, G.; Squarzanti, D.; Azzimonti, B.; Rolla, R.; Savoia, P.; Tarocchi, M.; et al. Effects of Probiotics Administration on Human Metabolic Phenotype. *Metabolites* **2020**, *10*, doi:10.3390/metabo10100396.
31. Khare, A.; Gaur, S. Cholesterol-Lowering Effects of Lactobacillus Species. *Curr. Microbiol.* **2020**, *77*, 638–644, doi:10.1007/s00284-020-01903-w.
32. Fang, T.; Guo, J.; Lin, M.; Lee, M.; Chen, Y.; Lin, W. Protective effects of Lactobacillus plantarum against chronic alcohol-induced liver injury in the murine model. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 8597–8608, doi:10.1007/s00253-019-10122-8.
33. Chapman, C.; Gibson, G.; Rowland, I. *In vitro* evaluation of single- and multi-strain probiotics: Inter-species inhibition between probiotic strains, and inhibition of pathogens. *Anaerobe* **2012**, *18*, 405–413, doi:10.1016/j.anaerobe.2012.05.004.
34. Torriani, S.; Felis, G.E.; Dellaglio, F. Differentiation of Lactobacillus plantarum, L. pentosus, and L. paraplantarum by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. *Appl. Environ. Microbiol.* **2001**, *67*, 3450–3454.
35. Song, Y.L.; Kato, N.; Liu, C.X.; Matsumiya, Y.; Kato, H.; Watanabe, K. Rapid identification of 11 human intestinal Lactobacillus species by multiplex PCR assays using group-and

species-specific primers derived from the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiol. Lett.* **2000**, *187*, 167–173.
36. Warke, R.; Kamat, A.; Kamat, M.; Thomas, P. Incidence of pathogenic psychrotrophs in ice creams sold in some retail outlets in Mumbai, India. *Food Control* **2000**, *11*, 77–83, doi:10.1016/S0956-7135(99)00027-4.
37. Charteris, W.; Kelly, P.; Morelli, L.; Collins, J. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. *J. Appl. Microbiol.* **1998**, *84*, 759–768.
38. Jensen, H.; Grimmer, S.; Naterstad, K.; Axelsson, L. *In vitro* testing of commercial and potential probiotic lactic acid bacteria. *Int. J. Food Microbiol.* **2012**, *153*, 216–222, doi:10.1016/j.ijfoodmicro.2011.11.020.
39. Commission Directive EU 6/2010 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards mercury, free gossypol, nitrites and Mowrah, Bassia, Madhuca (Text with EEA relevance). *Off. J. Eur. Uni.* **2002**, *140*, 1–10.
40. NRC. *Nutrient Requirements of Swine*, 7th ed.; National Research Council: Washington, DC, USA, The National Academies Press: Washington, DC, USA, 2012.
41. AOAC. *Official Methods of Analysis*, 21th ed.; Association of Official Analytical Chemists: Washington, DC, USA, 2019.
42. Christie, W.W.; Han, X. Lipid Analysis, Isolation, Separation, Identification and Lipidomic Analysis. *Oily Press Lipid Libr. Ser.* **2012**, 181–211, doi10.1533/9780857097866.181.
43. Shewale, R.N.; Sawale, P.D.; Khedkar, C.; Singh, A. Selection criteria for probiotics: A review. *Int. J. Probiotics Prebiotics* **2014**, *9*, 17.
44. Yun, J.H.; Lee, K.B.; Sung, Y.K.; Kim, E.B.; Lee, H.G.; Choi, Y.J. Isolation and characterization of potential probiotic lactobacilli from pig feces. *J. Basic Microbiol.* **2009**, *49*, 220–226.
45. Lukacova, D.; Karovicova, J.; Greifova, M.; Greif, G.; Sovcikova, A.; Kohajdova, Z. *In vitro* testing of selected probiotic characteristics of Lactobacillus plantarum and Bifidobacterium longum. *J. Food Nutr. Res.* **2006**, *45*, 77–83.
46. Singh, T.; Kaur, G.; Malik, R.; Schillinger, U.; Guigas, C.; Kapila, S. Characterization of Intestinal Lactobacillus reuteri Strains as Potential Probiotics. *Probiotics Antimicrob. Proteins* **2012**, *4*, 47–58, doi:10.1007/s12602-012-9090-2.
47. Vamanu, E. Effect of gastric and small intestinal digestion on lactic acid bacteria activity in a GIS1 simulator. *Saudi J. Biol. Sci.* **2017**, *24*, 1453–1457, doi:10.1016/j.sjbs.2015.06.028.
48. Bove, P.; Russo, P.; Capozzi, V.; Gallone, A.; Spano, G.; Fiocco, D. Lactobacillus plantarum passage through an oro-gastro-intestinal tract simulator: Carrier matrix effect and transcriptional analysis of genes associated to stress and probiosis. *Microbiol. Res.* **2013**, *168*, 351–359, doi:10.1016/j.micres.2013.01.004.
49. Suo, C.; Yin, Y.; Wang, X.; Lou, X.; Song, D.; Wang, X.; Gu, Q. Effects of Lactobacillus plantarum ZJ316 on pig growth and pork quality. *BMC Vet. Res.* **2012**, *8*, 1–12.
50. Betancur, C.; Martínez, Y.; Merino-Guzman, R.; Hernandez-Velasco, X.; Castillo, R.; Rodríguez, R.; Tellez-Isaias, G. Evaluation of oral administration of Lactobacillus plantarum CAM6 strain as an alternative to antibiotics in weaned pigs. *Animals* **2020**, *10*, 1218.
51. Wang, M.; Wu, H.; Lu, L.; Jiang, L.; Yu, Q. Lactobacillus reuteri promotes intestinal development and regulates mucosal immune function in newborn piglets. *Front. Vet. Sci.* **2020**, *7*, 42.

52. Wang, S.; Yang, L.; Tang, X.; Cai, L.; Liu, G.; Kong, X.; Blachier, F.; Yin, Y. Dietary supplementation with high-dose Bacillus subtilis or Lactobacillus reuteri modulates cellular and humoral immunities and improves performance in weaned piglets. *J. Food Agric. Environ.* **2011**, *9*, 181–187.
53. EFSA; Rychen, G.; Aquilina, G.; Azimonti, G.; Bampidis, V.; Bastos, M.d.L.; Bories, G.; Chesson, A.; Cocconcelli, P.S.; Flachowsky, G. EFSA Panel on Additives and Products or Substances used in Animal Feed: Guidance on the assessment of the efficacy of feed additives. *EFSA J.* **2018**, *16*, e05274.
54. Wu, Y.; Zhu, C.; Chen, Z.; Chen, Z.; Zhang, W.; Ma, X.; Wang, L.; Yang, X.; Jiang, Z. Protective effects of Lactobacillus plantarum on epithelial barrier disruption caused by enterotoxigenic Escherichia coli in intestinal porcine epithelial cells. *Vet. Immunol. Immunopathol.* **2016**, *172*, 55–63.
55. Huang, C.; Qiao, S.; Li, D.; Piao, X.; Ren, J. Effects of Lactobacilli on the performance, diarrhea incidence, VFA concentration and gastrointestinal microbial flora of weaning pigs. *Asian-Australas J. Anim. Sci.* **2004**, *17*, 401–409.
56. Pieper, R.; Janczyk, P.; Schumann, R.; Souffrant, W. The intestinal microflora of piglets around weaning with emphasis on lactobacilli. *Arch. Zootech.* **2006**, *9*, 28–40.
57. De Angelis, M.; Siragusa, S.; Caputo, L.; Ragni, A.; Burzigotti, R.; Gobbetti, M. Survival and persistence of Lactobacillus plantarum 4.1 and Lactobacillus reuteri 3S7 in the gastrointestinal tract of pigs. *Vet. Microbiol.* **2007**, *123*, 133–144.
58. Castillo, M.; Martín-Orúe, S.M.; Manzanilla, E.G.; Badiola, I.; Martín, M.; Gasa, J. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet. Microbiol.* **2006**, *114*, 165–170.
59. Dong, X.; Zhang, N.; Zhou, M.; Tu, Y.; Deng, K.; Diao, Q. Effects of dietary probiotics on growth performance, faecal microbiota and serum profiles in weaned piglets. *Anim. Prod. Sci.* **2014**, *54*, 616–621.
60. Wang, W.; Chen, J.; Zhou, H.; Wang, L.; Ding, S.; Wang, Y.; Song, D.; Li, A. Effects of microencapsulated Lactobacillus plantarum and fructooligosaccharide on growth performance, blood immune parameters, and intestinal morphology in weaned piglets. *Food Agric. Immunol.* **2018**, *29*, 84–94.
61. Naqid, I.A.; Owen, J.P.; Maddison, B.C.; Gardner, D.S.; Foster, N.; Tchórzewska, M.A.; La Ragione, R.M.; Gough, K.C. Prebiotic and probiotic agents enhance antibody-based immune responses to Salmonella Typhimurium infection in pigs. *Anim. Feed Sci. Technol.* **2015**, *201*, 57–65.
62. Belik, S.N.; Gorlov, I.F.; Slozhenkina, M.I.; Zlobina, E.Y.; Pavlenko, A.S. Morpho-functional state of the liver of the rats fed the rations with meat of the pigs grown with antimicrobials. *Pak. Vet. J.* **2015**, *35*, 325–328.
63. Klem, T.B.; Bleken, E.; Morberg, H.; Thoresen, S.I.; Framstad, T. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. *Vet. Clin. Pathol.* **2010**, *39*, 221–226.
64. Friendship, R.M.; Lumsden, J.H.; McMillan, I.; Wilson, M.R. Hematology and Biochemistry Reference Values for Ontario Swine. *Can. J. Comp. Med.* **1984**, *48*, 390–393.
65. Caprarulo, V.; Hejna, M.; Giromini, C.; Liu, Y.; Dell'Anno, M.; Sotira, S.; Reggi, S.; Sgoifo-Rossi, C.A.; Callegari, M.L.; Rossi, L. Evaluation of Dietary Administration of Chestnut and Quebracho Tannins on Growth, Serum Metabolites and Fecal Parameters of Weaned Piglets. *Animals* **2020**, *10*, 1945.

66. IZSLER. Parametri di Chimica Clinica: Valori Osservati in Suini di Diversa Età. Available online: https://www.izsler.it/pls/izs_bs/v3_s2ew_consultazione.mostra_pagina?id_pagina=1494 (accessed on 18 March 2021).
67. Pereg, D.; Kotliroff, A.; Gadoth, N.; Hadary, R.; Lishner, M.; Kitay-Cohen, Y. Probiotics for patients with compensated liver cirrhosis: A double-blind placebo-controlled study. *Nutrition* **2011**, *27*, 177–181, doi:10.1016/j.nut.2010.01.006.
68. Metzler, B.; Mosenthin, R. A review of interactions between dietary fiber and the gastrointestinal microbiota and their consequences on intestinal phosphorus metabolism in growing pigs. *Asian-Australas. J. Anim. Sci.* **2008**, *21*, 603–615.
69. Wang, J.; Wu, C.; Feng, J. Effect of dietary antibacterial peptide and zinc-methionine on performance and serum biochemical parameters in piglets. *Czech J. Anim. Sci.* **2011**, *56*, 30–36, doi:10.17221/341/2009-CJAS.
70. Li, C.; Nie, S.P.; Ding, Q.; Zhu, K.X.; Wang, Z.J.; Xiong, T.; Gong, J.; Xie, M.Y. Cholesterol-lowering effect of Lactobacillus plantarum NCU116 in a hyperlipidaemic rat model. *J. Funct. Foods* **2014**, *8*, 340–347.
71. Taranto, M.P.; Medici, M.; Perdigon, G.; Holgado, A.R.; Valdez, G. Effect of Lactobacillus reuteri on the prevention of hypercholesterolemia in mice. *J. Dairy Sci.* **2000**, *83*, 401–403.
72. Park, Y.; Kim, J.; Shin, Y.; Kim, S.; Whang, K. Effect of dietary inclusion of Lactobacillus acidophilus ATCC 43121 on cholesterol metabolism in rats. *J. Microbiol. Biotechnol.* **2007**, *17*, 655–662.
73. Xie, N.; Cui, Y.; Yin, Y.; Zhao, X.; Yang, J.; Wang, Z.; Fu, N.; Tang, Y.; Wang, X.; Liu, X.; et al. Effects of two Lactobacillus strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet. *BMC Complementary Altern. Med.* **2011**, *11*, doi:10.1186/1472-6882-11-53.

5 Effects of feed additives on gut microbiota modulation



The optimization of gut health is crucial for supporting animal performance and farm profitability. Even if a clear definition of healthful gut has not been formulated yet, it has been recognized that a strong relationship between a "healthy" gut and animal performance exists. Basic functions of intestine include nutrient digestion and absorption, energy production, microbiome integration, barrier function and mucosal immune response and withstand infections and non-infectious stressors. The intestinal health involves not only the gut homeostasis, but also other organs which support host ability to resilience towards environmental and microbiological stressors (Kogut, 2019). Nowadays, the understanding of microbiome-host interaction and the possibility to modulate either for positively impacting animal health and performance has gained pivotal role. Even if these two words are often used as synonyms, microbiome and microbiota have completely different meaning. Microbiota refers only to microorganisms that harbour a particular environment (e.g., gut) including bacteria, protozoa, viruses and fungi. Microbiome is meant as the entire genome of microbial populations including plasmids (Roto et al., 2015).

GIT health is a complex network between all physical, physiological, and microbiological functions (Kiarie et al., 2013). Intestine is the largest organ considering the surface area with a constant interaction with the environment. Therefore, the barrier function is fundamental for protecting the host form toxic compounds and potential pathogens invasion, contemporarily providing an efficient nutrient digestion and absorption. Another important intestinal function is related to the immune response. The mucosal surface provides a platform for microbial colonisation which forms a barrier against pathogens and regulates the immune development and producing metabolites for host (Kogut, 2019). The gut microbiota evolves within the host and has an important impact on each component which aids the support of intestinal health. Microbiota participate to the digestion and fermentation of undigestible molecules in feed and produces SCFA, essential ammino acids and vitamins that can nourish the host (Roto et al., 2015). Benefits related to microbiota modulation can play a role for counteracting the oxidative stress and enhancing mucosal morphology. The use of functional feed additives to modulate the intestinal microbiota can be essential for supporting animal health, improving oxidative stress, immune status and intestinal morphology (Moita et al., 2021).

The better understanding of how feed additives can modulate the gut microbiota could allow to establish nutritional strategies to decrease gastrointestinal pathologies incidence thus reducing antimicrobial treatments in livestock. For these reasons, after the encouraging results observed from *in vivo* trials tannin extracts, leonardite, tributyrin, *L. plantarum* and *L. reuteri* were evaluated for their effect on gut microbiota modulation.

References

Kiarie, E., Romero, L.F., Nyachoti, C.M., 2013. The role of added feed enzymes in promoting gut health in swine and poultry. Nutrition research reviews 26, 71-88.

Kogut, M.H., 2019. The effect of microbiome modulation on the intestinal health of poultry. Animal feed science and technology 250, 32-40.
Moita, V.H.C., Duarte, M.E., da Silva, S.N., Kim, S.W., 2021. Supplemental effects of functional oils on the modulation of mucosa-associated microbiota, intestinal health, and growth performance of nursery pigs. Animals 11, 1591.
Roto, S.M., Rubinelli, P.M., Ricke, S.C., 2015. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. Frontiers in Veterinary Science 2, 28.

PLOS ONE

RESEARCH ARTICLE

A mixture of quebracho and chestnut tannins drives butyrate-producing bacteria populations shift in the gut microbiota of weaned piglets

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Brief Introduction to the Study:
The following paper was based on encouraging results observed from the *in vivo* trial on the supplementation of chestnut and quebracho tannins extract in piglets. This study represents the first paper that evaluated the intestinal microbiota after dietary supplementation of tannins from chestnut and quebracho. These findings importantly contributed to the knowledge related to microbiota modulation of feed additives. Most important findings indicated that quebracho and chestnut tannins enhance the alpha diversity and can promote the butyrate producing bacteria, thus positively contributing to eubiosis.

5.1 A mixture of quebracho and chestnut tannins drives butyrate-producing bacteria populations shift in the gut microbiota of weaned piglets

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Abstract

Weaning is a critical period for piglets, in which unbalanced gut microbiota and/or pathogen colonisation can contribute to diseases that interfere with animal performance. Tannins are natural compounds that could be used as functional ingredients to improve gut health in pig farming thanks to their antibacterial, antioxidant, and antidiarrhoeal properties. In this study, a mixture of quebracho and chestnut tannins (1.25%) was evaluated for its efficacy in reducing the negative weaning effects on piglet growth. Microbiota composition was assessed by Illumina MiSeq 16S rRNA gene sequencing of DNA extracted from stools at the end of the trial. Sequence analysis revealed an increase in the genera *Shuttleworthia*, *Pseudobutyrivibrio*, *Peptococcus*, *Anaerostipes*, and *Solobacterium* in the tannin-supplemented group. Conversely, this dietary intervention reduced the abundance of the genera *Syntrophococcus*, *Atopobium*, *Mitsuokella*, *Sharpea*, and *Prevotella*. The populations of butyrate-producing bacteria were modulated by tannin, and higher butyrate concentrations in stools were detected in the tannin-fed pigs. Co-occurrence analysis revealed that the operational taxonomic units (OTUs) belonging to the families *Veillonellaceae*, *Lachnospiraceae*, and *Coriobacteriaceae* occupied the central part of the network in both the control and the tannin-fed animals. Instead, in the tannin group, the OTUs belonging to the families *Acidaminococcaceae*, *Alcaligenaceae*, and *Spirochaetaceae* characterised its network, whereas Family XIII Incertae Sedis occupied a more central position than in the control group. Conversely, the presence of *Desulfovibrionaceae* characterised the network of the control group, and this family was not present in the network of the tannin group. Moreover, the prediction of metabolic pathways revealed that the gut microbiome of the tannin group possessed an enhanced potential for carbohydrate transport and metabolism, as well as a lower abundance of pathways related to cell wall/membrane/envelope biogenesis and inorganic ion transport. In conclusion, the tested tannins seem to modulate the gut microbiota, favouring groups of butyrate-producing bacteria.

Introduction

Tannins are natural polyphenolic compounds that are widely distributed in plants, mostly in the wood of several tree species. Because of their heterogeneous composition and chemical properties, tannins are divided into five categories, namely gallotannins, ellagitannins, complex tannins, phlorotannins and condensed tannins [1]. The need to reduce the use of antibiotics as a prophylaxis mean to treat post-weaning diarrhoea prompted researchers to search for alternatives. Tannins could represent a natural alternative to antibiotics, and their biological activities make them suitable for that

purpose [2]. Tannins from chestnut (*Castanea vesca*) are frequently used as feed supplements for pigs, whereas quebracho (*Schinopsis lorentzii*) tannins or mixtures of both have been evaluated in other animal species, such as poultry, sheep, rabbits, and cows [3–6]. Tannins from chestnut are defined as hydrolysable, while those from quebracho are categorised as condensed. This classification is based on their sensitivity to hydrolysis, the type of monomer unit or the degree of polymer condensation [7].

Traditionally, tannins were regarded as anti-nutritional factors in monogastric and ruminant farm animals responsible for reduced feed digestibility and worse animal performance [8,9]. Thereafter, some studies have shown that tannins can be used in ruminants and monogastrics, particularly in pigs, without any detrimental effects on animal growth. Chestnut tannins significantly improved feed efficiency and growth performance when supplemented in the diet of weaned piglets [10]. Galassi et al. [11] also reached the same conclusion using chestnut tannins as a dietary supplement in heavy pigs since no anti-nutritional effects were observed. However, animals might not find tannin-containing feed palatable due to its astringent taste. Thus, it is crucial to establish the correct percentage of tannins to add to the diet in order to avoid a reduction in feed intake. Notably, in monogastric animals, as compared with ruminants, the dosage can be reduced and still have positive effects. Apart from these application problems, tannins are recognised to have antimicrobial, antioxidant, and antidiarrhoeal properties [12–16]. These properties can be extremely useful in the weaning of piglets, which represents a critical period in the animal's life. Indeed, several types of stress can be caused by the removal of piglets from their mother, the sudden change in their diet, and the crowding of the farm environment. All these factors can contribute to the development of diarrhoea and a slowdown in growth. In particular, diarrhoea can be due to an unbalanced gut microbiota caused by the introduction of solid feed [17] or the colonisation of pathogenic agents [18–20]. Indeed, encouraging results have been obtained using chestnut tannins to prevent diarrhoea in piglets infected with an *Escherichia coli* ETEC strain. The feed supplementation reduced the incidence and severity of diarrhoea with no negative effects on animal performance [21]. Chestnut tannins were also used as an alternative to zinc oxide in weaning piglets, resulting in improved animal health and a reduction in diarrhoea by means of multifactorial mechanisms of action [22]. In the same study, the authors reported increased levels of butyrate and propionate in the colon as a consequence of a higher amount of indigested carbohydrates reaching the distal gut.

Concerning the effect of tannins on the gut microbiota, very few data are available in the literature. Classical microbiological techniques have been used to investigate several groups of bacteria that are considered important for animal health [10]. We found only one study that considered tannins extracted from grapes, showing

that the supplemented diet increased the abundance of *Lachnospiraceae*, *Clostridiales*, *Lactobacillus*, and *Ruminococcacceae* [23]. These groups of bacteria are recognised as major producers of short-chain fatty acids (SCFAs) in the human and animal guts. In particular, *Lachnospiraceae* and *Clostridiales* include the most important producers of butyric acid [24–26]. Butyrate plays an important role in the health of piglets during weaning, reducing intestinal inflammation and favouring the adaptation of the intestine to the change in diet [27]. Moreover, a reduction in butyrate-producing bacteria can promote the growth of pathogenic bacteria, such as *Salmonella* [28]. Thus, an increase in butyrate in the gut of weaning piglets could be an important factor in mediating the positive effects of tannins on animal health.

In a recent study, we evaluated tannins derived from chestnut and quebracho trees were evaluated for their capability to affect growth performance, blood metabolic profile, and faecal nitrogen concentration in post-weaned piglets [29]. Not surprisingly, we observed a higher fecal nitrogen excretion in tannin-fed piglets consistently with the known tannin property to form less digestible complexes with dietary proteins [30]. However, the performance of treated animals was comparable with that of controls, despite a very high dose of tannins administered. This result, in addition to the lower serum urea concentration detected in tannin-fed piglets, suggested a putative role of the gut microbiota in improving protein digestion and nitrogen utilization thereby supporting overall animal health. Therefore, in the present study we sought to highlight microbial changes occurring in the gut of weaned piglets that could mitigate the adverse effects of tannins on feed utilisation.

Materials and Methods

Animals and sample collection

The present study was undertaken as part of an investigation of the effects of tannins on piglet gut microbiota composition and performance. A detailed description of the experimental animals, study design, and dietary treatments can be found in our previous study [29]. The trial was conducted in agreement with the Italian regulations on animal experimentation and ethics (DL 26/2014) and with the European regulation (Dir. 2010/6). Moreover, the trial was approved by the Animal Welfare Body of the University of Milan (number 31/2019). Briefly, a total of 120 weaned piglets (28 ± 2 days age; 50% female and 50% male), were allotted in randomized complete block design into two experimental groups: control group (CTR) and treatment group (TAN). There were 60 pigs per treatment with 6 replicate pens and 10 pigs per pen. The groups were homogeneous in terms of gender, weight and litter. After one week of adaptation (considered day 0, piglets were 35 days old), during which all animals

received the same basal diet, the experimental diets were distributed *ad libitum* to all animals for 40 days. Experimental diets (Plurimix, Fabermatica, CR, Italy) were formulated according to animal requirements for the post-weaning phase (Ferraroni Mangimi SpA, Bonemerse, Italy). The two diets were isoenergetic and isoproteic and differed for the inclusion of 1.25% of tannin extract (contain 75 g of tannin/100 g of dry matter; Ch/Qu; Silvafeed Nutri P/ENC for Swine, Silvateam, Italy) from chestnut and quebracho trees in the treatment group. Both diets fulfilled the National Research Council (2012) [31] requirements for post-weaned piglets (S1 Table). Twelve piglets (2 piglets per pen) were randomly selected from each of the two dietary groups. During the entire experimental period, mortality was registered, and the incidence of diarrhea was calculated based on the number of piglets with clinical sign of diarrhea. Stools were individually collected from rectal ampulla at the end of the trial, which corresponded to day 40 of the dietary intervention (75-day-old piglets). Samples were immediately frozen in dry ice and then transferred to the laboratory for further analysis.

Gas chromatographic analysis of faecal short-chain fatty acids

The extraction of short-chain fatty acids (SCFAs) was carried out starting with a sample of 3 g resuspended in 9 ml of distilled water by stirring. The suspension was centrifuged at 2000 rpm for 15 min, and then 2 ml of the obtained supernatant was added to 1 ml of a pivalic acid solution (internal standard, 1 g L^{-1} in distilled water) and 1 ml of a 0.12Moxalic acid solution. After mixing, the suspension was centrifuged at 2000 rpm, and the resulting upper phase was microfiltered in vials.

Gas chromatographic analysis was carried out in a Shimadzu 2025 gas chromatograph equipped with an AOC-20i auto-sampler (Shimadzu Srl, Milan, Italy), FID detector, using a 30 m x 0.250 mm x 0.25 μm DB-FFAP capillary column (Agilent Technologies, Inc. Santa Clara, CA, USA). The temperatures of the injector and detector were 200 and 220 ˚C, respectively. The injection (1 μl of the sample) was carried out in split mode. The analysis was performed at a constant flow of carrier gas using the following cycle of temperature. The initial temperature of 60 ˚C was held for 5 min; the temperature was then raised to 160 ˚C at 5 ˚C min^{-1} and finally to 190 ˚C at 10 ˚C min^{-1}, and this last temperature was held for 7 min.

Data acquisition and processing were performed using the LabSolutions Lite software (v5.82, Shimadzu Srl, Milan, Italy). SCFA identification was based on the retention time of an external standard, while pivalic acid was used as an internal standard.

Bacterial DNA extraction, V3-V4 region amplification, and sequencing

Bacterial genomic DNA was extracted from 50 mg (wet weight) faecal samples using the FastDNA ™ SPIN Kit for Soil (MP Biomedicals, Switzerland) following the manufacturer's instructions. Quantification of the extracted DNA was carried out by a Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA), and the quality check was performed using agarose gel electrophoresis. Amplification of the V3-V4 regions of the bacterial 16S rRNA gene was obtained using the primers 343F and 802R following the already described procedures [32]. The PCR products were checked by agarose gel electrophoresis and quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA) in order to prepare a pool of amplicons in which the PCR products of each sample were present in equimolar concentrations. The pool was then purified using a DNA Clean & Concentrator ™-5 Kit (Zymo Research, Irvine, CA, USA). Fasteris SA (Geneva, Switzerland) performed the sequencing of amplicons using Illumina's MiSeq v3 platform in 2 x 300 bp mode. Trimmomatic version 0.32 (http://www.usadellab.org/cms/index.php?page=trimmomatic) was used for quality filtering of raw reads (quality score ≥ 30) by sliding window trimming (window size: 4 base, quality: 15) and by dropping reads below a specified length (60 bases) [33]. Overlapped reads were mapped against the SILVA database (Version SSUref_NR99_115_tax_silva_DNA.fasta) using Burrows-Wheeler Alignment Tool version 0.7.5a (http://bio-bwa.sourceforge.net/). The package SAM tools was used to merge alignments and compute the number of reads mapped onto each OTU [34]. The data of the 16S rRNA gene sequences are available at the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk) for tannin samples (accession numbers ERS5141801-ERS5141812) and control group samples (accession numbers ERS5141296-ERS5141307).

Statistical analysis

Statistical analysis of the microbiota data was performed using the MicrobiomeAnalyst tool [35,36]. This tool allowed the calculation of alpha diversity based on the Chao 1, observed species, Simpson, and Shannon metrics. Significant differences between these indices were calculated using a t-test. Beta diversity across samples was calculated using the Bray–Curtis index and the PERMANOVA statistical methods. The EdgeR algorithm with an adjusted pvalue cut-off, false discovery rate (FDR), of 0.05 was used to identify significant differences in taxa abundance of faecal bacteria between the two dietary groups. Concerning the prediction of the functional profiles of the bacterial communities across the two dietary groups, the operational taxonomic unit (OTU) table was uploaded in the MicrobiomeAnalyst software using

the Marker Data Profiling option. The aim was to generate a Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology (KO) assignment table using the Tax4Fun source in order to further analyse the data in the shotgun data profiling option. The differential relative abundance of genes across the two dietary groups was analysed using the EdgeR and Random Forest algorithms. Moreover, clusters of orthologous groups (COGs) of proteins were analysed using the same algorithms to identify the significant differences in COGs between the two dietary groups. Network analysis of faecal microbiota was performed using SparCC correlation coefficients as implemented in the R package SpiecEasi v1.1.0 [37] and visualised using qgraph v1.6.5 [38]. GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA) was used to perform t-test and Spearman's correlation analyses on the SCFA and serum urea data. For each genus, we examined the effect of the relative abundance on the recorded body weight. We fitted linear models separately for control and tannin-fed groups using body weight as response variable and the relative abundance of the focal genus as regressor. Further, the covariance between control and tannin-fed groups was evaluated including the interaction between relative abundance and group (control and tannin-fed animals) to the model. Analyses were carried out using R v4.0.2 [39].

Results and discussion

Microbiota composition and community diversity associated with tannin supplementation

A total of 2,694,143 reads were obtained after the filtering procedure, with an average sequence number for each sample of 112,255. As shown in the principal coordinate analysis (PCoA) ordination plot (Fig 1A), the tannin and control groups did not cluster separately, although PERMANOVA analysis indicated significant differences between the gut microbiota of piglets ($R2 = 0.24$, $p < 0.001$). To investigate the modification of the gut microbiota composition due to tannin supplementation, the alpha diversity of bacterial communities was calculated. The Chao 1 index did not differ between the control group (291.89 ± 41.8) and the tannin group (318.47 ± 23.0) ($p = 0.06$) (data not shown), whereas the observed species index was significantly higher in the tannin group (303.58 ± 23.1 vs. 269.83 ± 43.14, $p = 0.03$) (Fig 1B). The Shannon and Simpson indices, both reflecting the species richness and evenness, were significant higher in the tannin-supplemented animals (4.09 ± 0.21 vs. 3.56 ± 0.46, $p = 0.02$; 0.96 ± 0.01 vs. 0.93 ± 0.03, $p = 0.001$) (Fig 1C and 1D). In general, biologically diverse communities are thought to have high stability and resistance in the face of several disturbances and environmental changes [40]. Regarding the gut microbiota composition at the phylum level (Fig 2A), a significant increase in *Cyanobacteria* and *Spirochaetae* (FDR < 0.001 and FDR = 0.012, respectively) was detected in stools collected from the tannin-supplemented animals, whereas *Bacteroidetes* and *Actinobacteria* (FDR = 0.002 and FDR

= 0.02, respectively) were reduced (Fig 3A and S2 Table). So far, no data on piglet gut microbiota modulation associated with tannins are available in the literature; a reduction in the faecal levels of *Bacterioidetes* was detected in chickens fed on a diet enriched with chestnut tannins [41]. Moreover, at the family level, several differences were found between the two dietary groups of animals, as shown in Fig 2B.

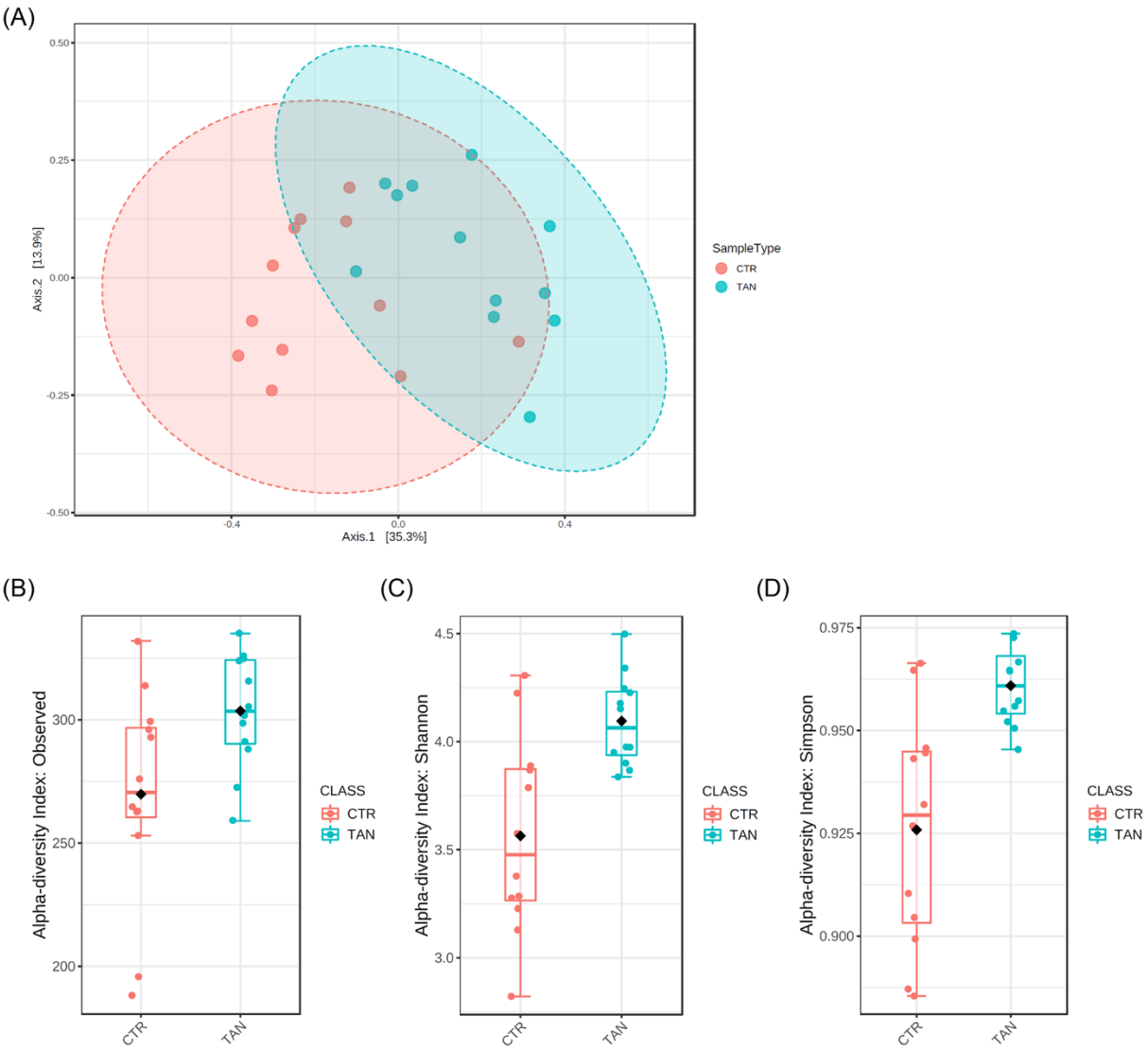

Fig 1. (A) Principal coordinate analysis (PCoA) plot based on Bray–Curtis distances of the intestinal microbial communities of weaning piglets fed on a diet with tannin (TAN) and the control group receiving a basal diet (CTR). (B) Observed species index (p = 0.03), (C) Shannon index (p = 0.02), and (D) Simpson index (p = 0.001). The index values are graphically presented by box plots, each of which represents the interquartile range, whereas the line inside the box represents the median.

Tannin supplementation strongly increased the relative abundance of *Peptococcaceae* and *Clostridiaceae*, whereas a reduction was observed in the relative abundance of *Prevotellaceae* and *Eubacteriaceae*. The microbiota of tannin-fed piglets had fewer sequences from *Coriobacteriaceae*, *Desulfovibrionaceae*, *Veillonellaceae*, *Rikenellaceae*, and *Deferribacteraceae*, whereas it was enriched in *Spirochaetaceae* and *Peptostreptococcaceae*

(Fig 3B and S2 Table). At the genus level, many significant differences (FDR<0.05) were found between the two dietary groups (Fig 2C). Therefore, only the most significant ones were taken into consideration, namely those showing an FDR <0.0001. In the tannin-supplemented animals, the

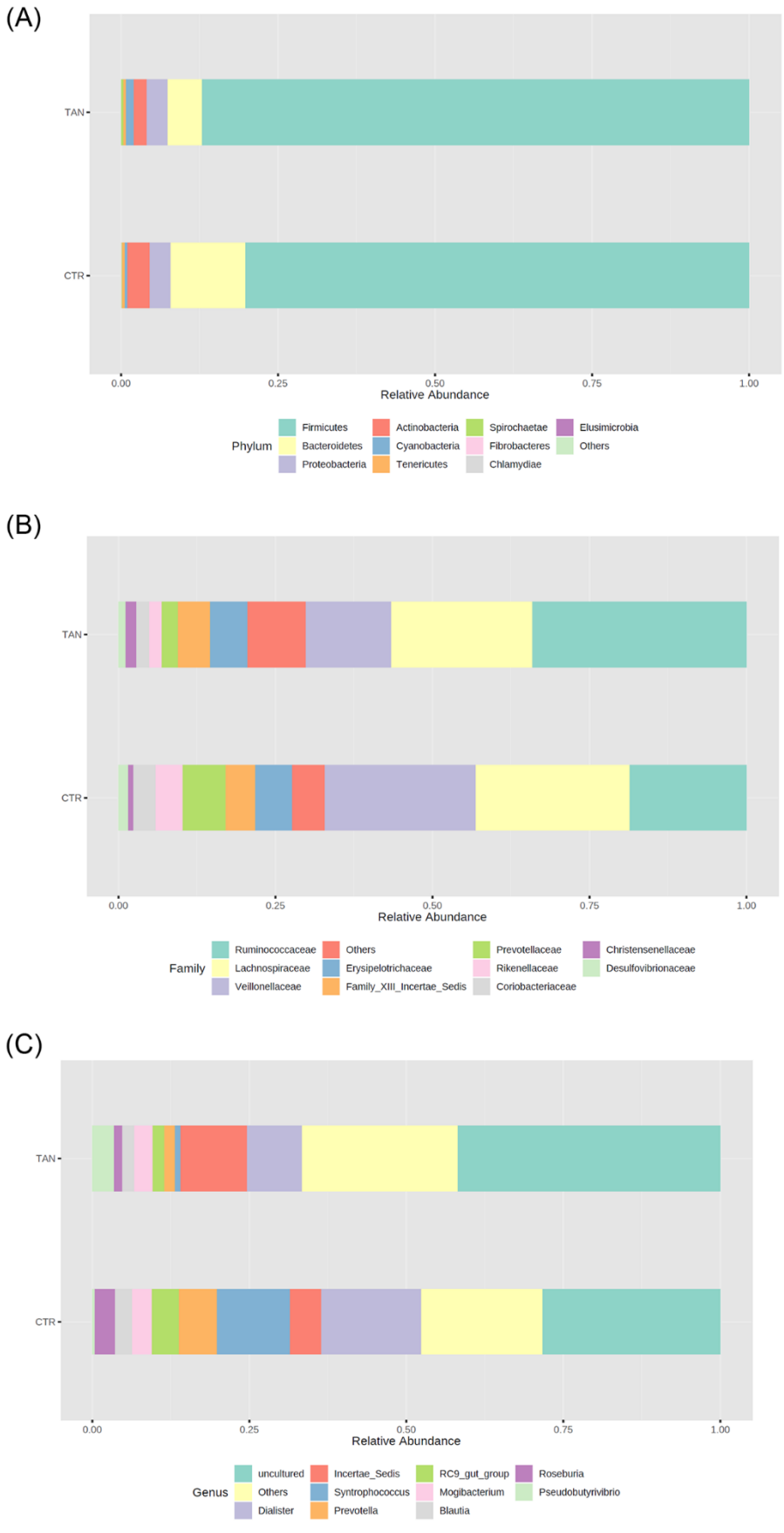

Fig 2. Relative abundances of phyla (A), families (B), and genera (C) observed in the tannin-supplemented group (TAN) compared with those of the control group (CTR). Only the top taxa are represented in the graphs.

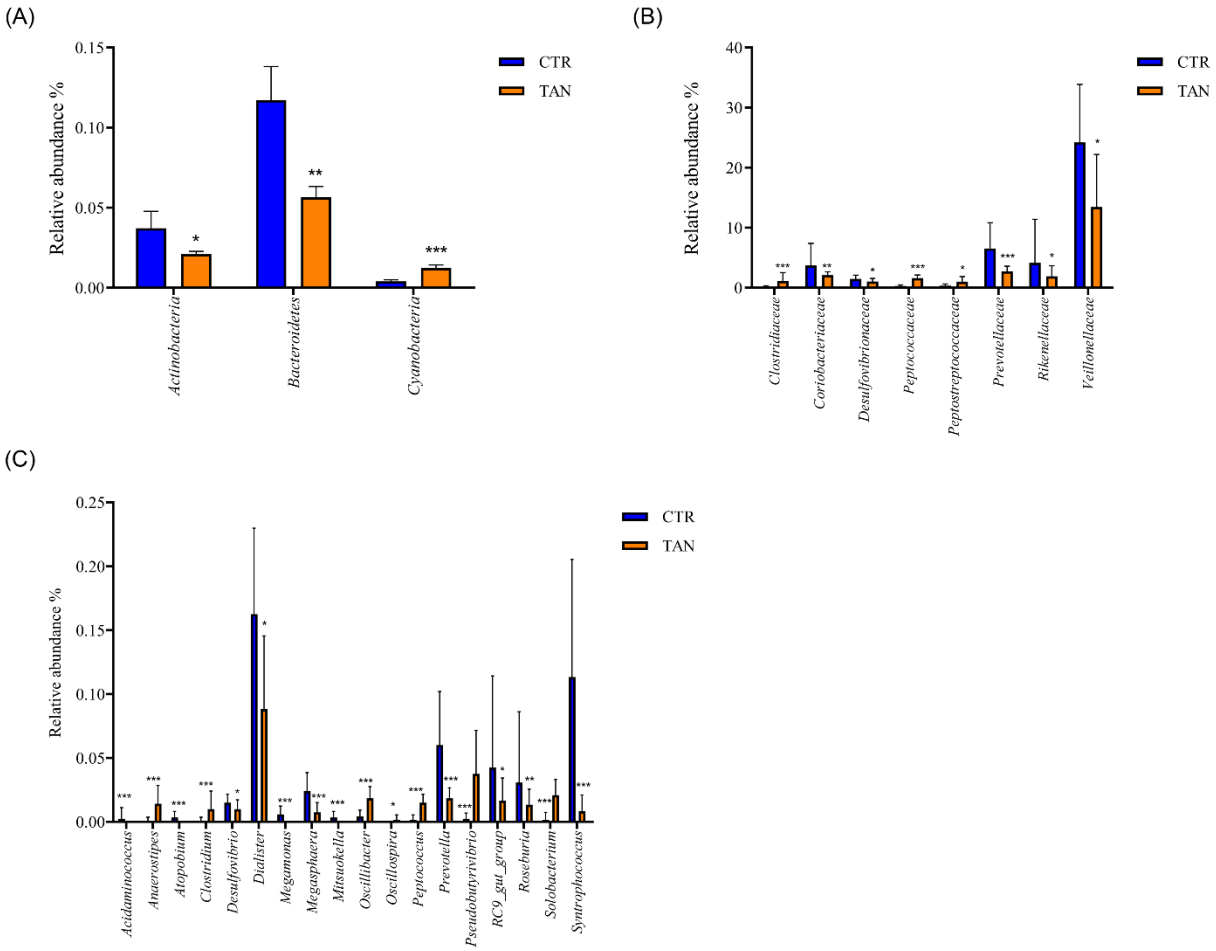

Fig 3. Relative abundance of fecal bacterial populations at phylum (A), family (B) and genus (C) levels in tannin-fed (TAN) and control (CTR) piglets. Only taxa with a mean relative abundance higher than 0.002% are shown. p value significance levels were reported as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

genera *Shuttleworthia*, *Pseudobutyrivibrio*, *Peptococcus*, *Anaerostipes*, and *Solobacterium* were significantly increased. Conversely, the genera *Syntrophococcus*, *Atopobium*, *Mitsuokella*, *Sharpea*, and *Prevotella* were significantly decreased in samples collected from the tannin-supplemented group of animals (Fig 3C and S3 Table).

Given the high number of statistically significant different taxa between the two dietary groups, a random forest analysis was performed to identify the bacterial populations that were important in differentiating the tannin-fed versus control animals. Random forest analysis revealed that *Peptococcaceae*, an unidentified member of the human gut metagenome, and *Prevotellaceae* were the most discriminant taxa at the family level, while *Peptococcus*, *Solobacterium*, *Syntrophococcus*, *Pseudobutyrivibrio*, and *Prevotella* had the highest importance scores at the genus level (Fig 4). Previous studies reported that *Peptococcus* was more abundant in pigs showing high performance, suggesting a positive correlation with body weight [42–44]. On the

other hand, in piglets, a higher risk of post-weaning diarrhoea has been associated with an increase in the intestinal levels of *Prevotella* concurrently with a decrease in non-pathogenic *Escherichia coli* and beneficial *Firmicutes* [45,46].

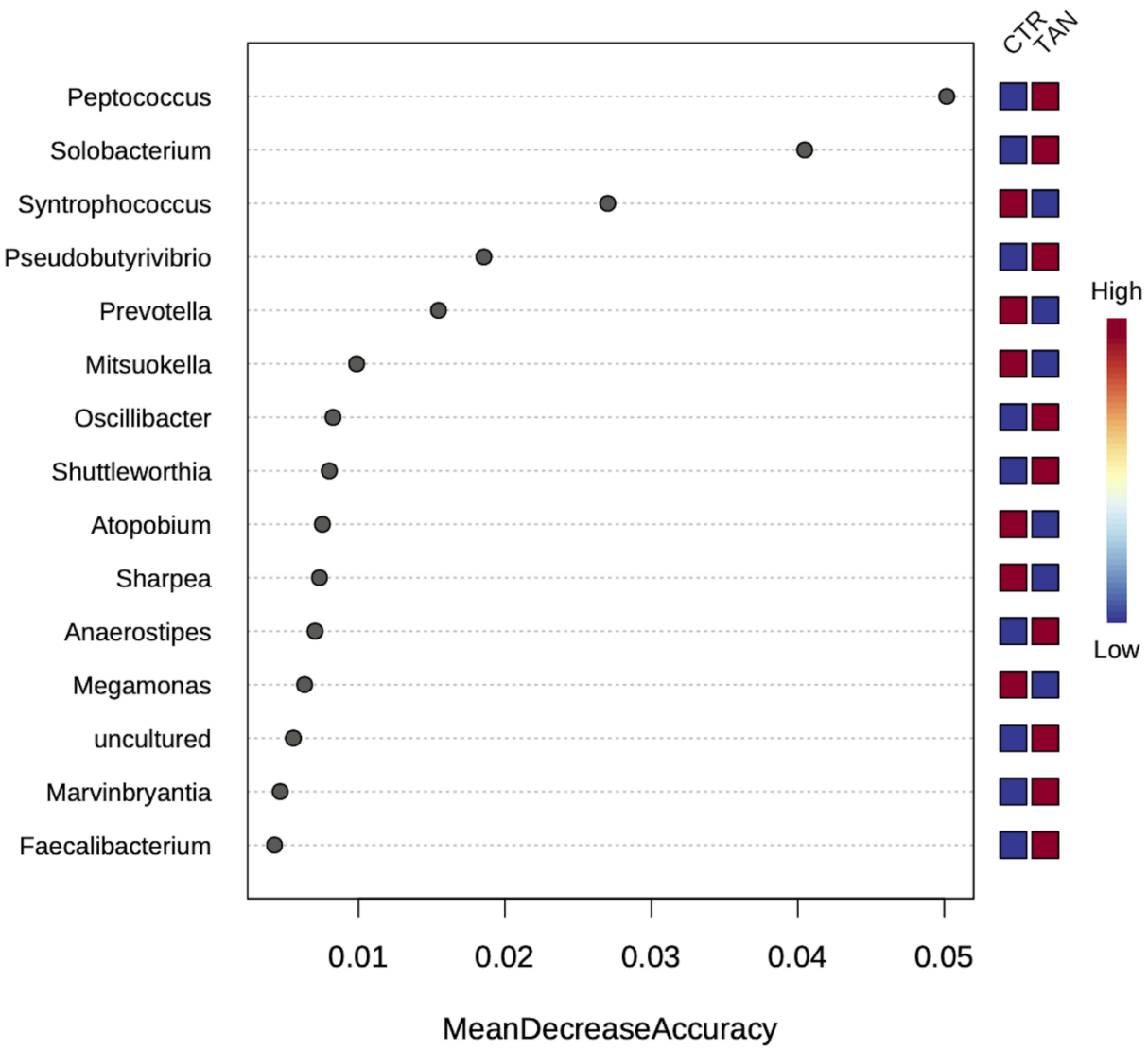

Fig 4. The most discriminant genera between the control- and tannin-sample sequences as sorted by Random Forest analysis using the mean decrease in accuracy.

According to our results, tannins could contribute to preventing an increase in *Prevotella* in the gut. In our experimental animals, we observed transient signs of diarrhoea after seven days of treatment; the highest incidence was detected at 14–28 days with slightly lower percentages in tannin-fed piglets vs controls, though such differences were not significant [29]. Other populations that were modulated by tannin included genera such as *Butyrivibrio*, *Pseudobutyrivibrio*, *Oscillospira*, and *Oscillibacter*. The genus *Butyrivibrio* has been detected only in animals showing high performance, suggesting that the high efficiency of this bacterial genus in degrading complex carbohydrates could positively impact animal feed conversion [47]. Regarding

Oscillibacter, this genus was correlated with a higher weight gain [47]. The genus *Oscillospira*, on the other hand, was positively associated with harder stools [48], whereas its decrease in human gut microbiota was correlated with an inflammatory status [49]. Both properties could have positive implications during the weaning period, so the potential impact of these bacterial populations on pig gut function and physiology warrants further investigation. Moreover, *Solobacterium* was increased in the tannin group, although it has been described as being associated with low feed intake in piglets [47]. Nevertheless, increased levels of this genus, together with other anaerobic bacteria, were correlated with positive effects in new-born piglets treated with antibiotics [50]. To measure the relationship between bacterial relative abundance and animal body weight for both control and tannin-fed groups, we fitted linear models to observed data. *Mucispirillum* was the only genus for which a significant interaction between the relative proportion and the experimental group was recorded ($p = 0.01$). The interaction effect in *Butyrivibrio* and *Oscillospira* tended to significance ($p \sim 0.07$). Notably, *Mucispirillum* showed significant association ($R = 0.88$, $p = 0.00017$) only within the tannin-fed group indicating that its reduction was related to a lower animal body weight. *Mucispirillum* is an inhabitant of the intestine of rodents and other animals, including pigs. It is considered an immunogenic commensal bacterium able to adhere to the intestinal mucus and to interact with the host immune system [51]. We used the Spearman's rank correlation coefficient to measure the association between plasma urea, nitrogen excretion and fecal bacterial genera. Correlation analysis revealed positive correlations between *Oscillospira* ($r = 0.74$, $p = 0.00004$), *Faecalibacterium* ($r = 0.64$, $p = 0.0008$), *Peptococcus* ($r = 0.69$, $p = 0.0002$), *Anaerostipes* ($r = 0.61$, $p = 0.0016$), *Solobacterium* ($r = 0.53$, $p = 0.007$) and nitrogen excretion. This parameter showed negative correlations with *Syntrophococcus* ($r = -0.59$, $p = 0.002$), *Prevotella* ($r = -0.54$, $p = 0.007$), *Mitsuokella* ($r = -0.51$, $p = 0.01$). The fundamental role of intestinal nitrogen in driving the interaction between the host and the gut microbiome has been already established [52]. Based on a predictive model analysis, nitrogen availability was reported to exert a primary control in the assembly of gut microbial communities by limiting microbial competition for carbon sources [52]. In addition, a negative correlation was found between urea and *Anaerostipes* ($r = -0.779$; $p<0.0001$), *Pseudobutyrivibrio* ($r = -0.687$; $p = 0.0002$), *Faecalibacterium* ($r = -0.500$; $p = 0.013$), *Oscillibacter* ($r = -0.436$; $p = 0.033$), *Oscillospira* ($r = -0.522$; $p = 0.009$), *Shuttleworthia* ($r = -0.484$; $p = 0.017$) and *Solobacterium* ($r = -0.543$; $p = 0.006$). All these taxa increased in tannin-fed piglets as compared with controls. Conversely, positive correlations were detected between this blood parameter and genera that were less abundant in tannin-fed animals, i.e. *Atopobium* (0.685; $p = 0.0002$), *Syntrophococcus* ($r = 0.580$; $p = 0.003$), *Acidaminococcus* ($r = 0.471$; $p = 0.02$), *Howardella* ($r = 0.545$; $p = 0.006$), *Megamonas* ($r =$

0.410; p = 0.046) and *Megasphaera* (r = 0.520; p = 0.009). Remarkably, a number of studies have reported that lower serum urea concentration is linked to a more efficient utilization of protein/nitrogen by animals [53,54]. Although correlation does not imply causation, the association between this marker and fecal bacterial taxa found in this the present study suggests a potential involvement of tannin-driven microbial population shifts in whole-animal nitrogen balance in piglets.

Short chain fatty acids content in stools

The determination of SCFAs in piglet stools revealed a significant increase in butyrate proportion in the tannin group (12.88 ± 3.11) as compared with the control group (9.93 ± 2.41, p = 0.018). In addition, a significant decrease in valerate (2.69 ± 0.62 vs. 3.51 ± 1.09, p = 0.044) was also detected in the tannin-fed animals (Fig 5). The total concentration of SCFA was similar (p = 0.1413) in both groups of experimental piglets (CTR 0.0723 mmol/g±0.014; TAN 0.082 mmol/g±0.017).

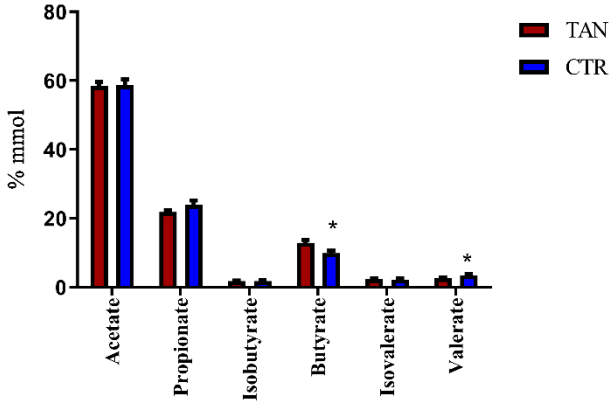

Fig 5. Proportion of short-chain fatty acids in control (CTR) and tannin-fed (TAN) piglets. The * indicates a p value ≤ 0.05.

The importance of butyrate for the host's gut health is well established, and its positive role in the intestinal mucosa and immune responses has been described in weaning piglets [27]. Results of the Spearman's correlation analysis indicated that there was a significant positive association between butyrate and *Pseudobutyrivibrio* (r = 0.669, p = 0.0003), *Atopobium* (r = -0.527, p = 0.008), and *Anaerostipes* (r = 0.509, p = 0.011). As concerns valerate, it can be produced by the degradation of lactate, as shown for some strains of *Megasphaera elsdenii* in pigs [55], but small amounts of this chain fatty acid can be formed by protein and amino acid degradation by other intestinal bacteria [53]. Based on our correlation results, fecal valerate content displayed a weak positive association with the levels of *Megasphaera* (r = 0.44, p = 0.031) and *Desulfovibrio* (r = 0.44 p = 0.034). *Megasphaera* showed the same positive correlation in the proximal colon of pigs fed a trans-glycosylated starch diet [56]. Overall, valerate has been poorly

investigated, but it has been described as an alternative source of energy for colonocytes in pigs [54].

Co-occurrence networks of the intestinal microbiota of control and tannin group animals

To explore the dynamics of gut bacterial interactions across diets, a bacterial community network analysis was performed for each group of animals (Fig 6A and 6B). Only significant correlations ($p < 0.05$) are shown.

In the network of the control group, *Desulfovibrionaceae*, *Veillonellaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Succinivibrionaceae*, *Erysipelotrichaceae*, and Family XIII Incertae Sedis were positively correlated with each other. The strongest correlations were found between *Veillonellaceae* and *Prevotellaceae* (SparCC = 0.86), between *Succinivibrionaceae* and *Desulfovibrionaceae* (SparCC = 0.81), and between *Succinivibrionaceae* and Family XIII Incertae Sedis (SparCC = 0.78). Conversely, *Christensenellaceae* showed strong negative correlations with *Lachnospiraceae* (SparCC = −0.89) and *Prevotellaceae* (SparCC = −0.94). At the edge of the network, Family XIII Incertae Sedis was negatively correlated with *Peptostreptococcaceae* (SparCC = −0.74), *Ruminococcaceae* (SparCC = −0.96), and *Campylobacteriaceae* (SparCC = −0.95). In the tannin group samples, the families *Veillonellaceae*, *Lachnospiraceae*, and *Coriobacteriaceae* occupy the central part of the network, as for the previous group. Moreover, the *Spirochaetaceae* family, which characterised the network of the tannin group, showed strong positive correlations with *Christensenellaceae* (SparCC = 0.88) and *Rikenellaceae* (SparCC = 0.87). The presence of *Acidaminococcaceae* and *Alcaligenaceae* characterised the co-occurrence network of the tannin group. *Veillonellaceae* showed strong positive correlations with both *Acidaminococcaceae* (SparCC = 0.85) and *Alcaligenaceae* (SparCC = 0.80). Finally, Family XIII Incertae Sedis was not present in the central part of the tannin group network and did not display any significant correlations with other families. These findings confirmed that tannin supplementation influenced both the composition of the gut microbiota of piglets and the interactions between different bacterial groups, mainly hydrogen-consuming populations such as *Desulfovibrio*. This decrease could be due to a particular sensitivity of this genus to tannins and/or its derivative compounds, as well as to a different hydrogen balance in the gut environment.

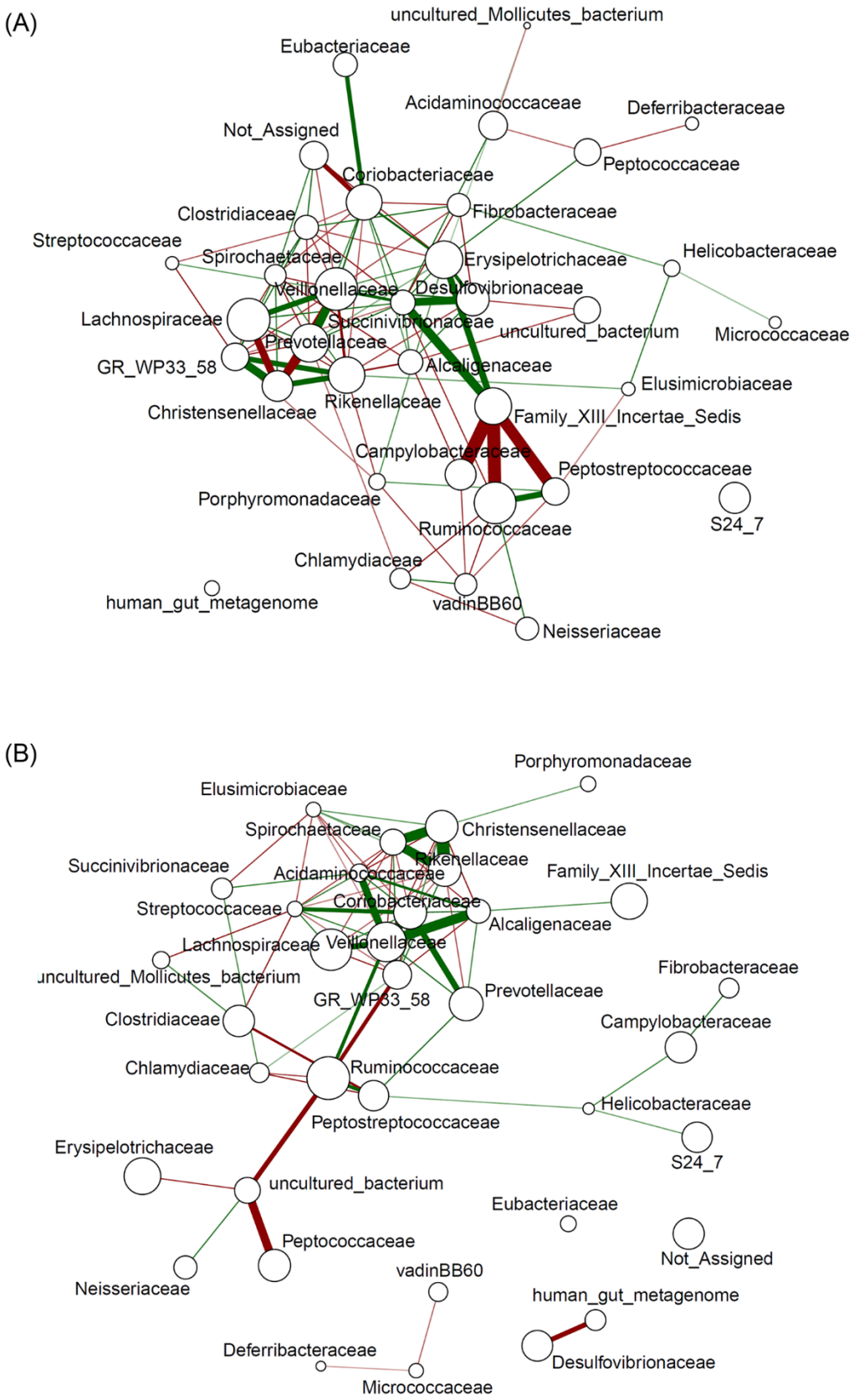

Fig 6. SparCC co-occurrence network analysis at the family level of the control (A) and the tannin (B) group microbiota. The node size represents the relative abundance of families. Lines represent correlations between the nodes they connect. The green colour of an edge indicates positive correlations, whereas the red colour denotes negative correlations. The colour intensity and line thickness indicate the correlation magnitude. Only significant correlations ($p < 0.05$) are shown.

Functional prediction

The COG functional category profiles were analysed to identify significant differences between the two groups of samples (Fig 7 and S4 Table). More specifically, a significant reduction in genes involved in lipopolysaccharide (LPS) biosynthesis (i.e. K00912, K00677, K02527, K00748, and K03272) was found in the tannin group. LPSs are components of the outer membrane of Gram-negative bacteria that are often associated with inflammation. They are able to induce inflammation by activating the host's immune cells [57], although LPSs seem to play a role in facilitating the host's tolerance of gut microbiota [58]. The reduction in LPS genes in our samples could be due to a decrease in Gram-negative bacteria numbers, such as *Bacteroidetes* and particularly *Prevotella*, following the supplementation of tannins. These results could suggest a reduction in components capable of inducing immune responses, and, consequently, of affecting the inflammation status in piglets fed with tannins. Further analysis is required in order to confirm this hypothesis. Moreover, in the tannin group samples, the genes encoding for enzymes associated with propanoate (i.e. K00169, K00170, K00172, and K01026) and butanoate (K00248, K14534, K00169, K00170, K04072, K00172, and K00171) metabolism were enriched. In particular, the enriched genes of the butanoate pathway were genes encoding for enzymes involved in carbohydrate fermentation. Conversely, genes involved in the butyrate conversion from succinate were less represented in the tannin group. For instance, one genus that was decreased in the tannin-supplemented group was *Prevotella*, a highly efficient fibre degrader producing succinate. Moreover, in the same group of samples, a reduction in genes encoding for phosphotransbutyrylase and butyrate kinase was detected, while genes encoding for butyryl-CoA: acetate-CoA transferase were enriched. Three of the genes that were enriched in the tannin samples encoded for common oxidoreductases in both the butanoate and the propanoate pathway. Overall, the outcome of the *in silico* analysis indicates a different balance of metabolic activities in the gut microbiota of animals fed on tannins, as a result of the modulation of bacterial populations that produce butyrate via different metabolic routes. Indeed, *Acidaminococcus*, *Megasphaera*, *Pseudoramibacter*, and *Roseburia* showed a statistically significant reduction, whereas *Shuttleworthia*, *Anaerostipes*, *Faecalibacterium*, *Pseudobutyrivibrio*, *Butyrivibrio*, *Oscillibacter*, and *Oscillospira* were increased in the tannin group [25,49,59,60]. Therefore, the species-specific functionalities and the intra-species diversity can explain the modulation of butyrate metabolism.

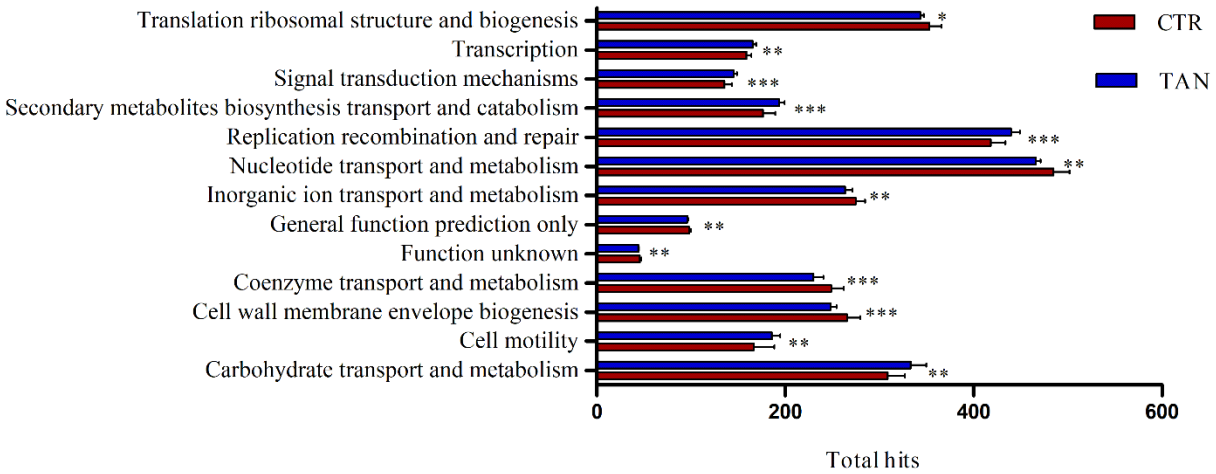

Fig 7. Significant different orthologous groups of proteins between the tannin (TAN) and control (CTR) piglets. p value significance levels were reported as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Notably, the dietary regulation of the metabolic pathways underlying butyrate production in tannin-fed piglets seems to result in an overall positive balance, as indicated by the higher production yield of butyric acid in the tannin group as described above. Moreover, our analysis revealed a significant increase in carbohydrate metabolism that has been associated with low feed efficient pigs [47,61].

Conclusion

As far as we know, this is the first investigation of the effects of quebracho and chestnut tannins on the gut microbiota of weaned piglets. In this study, tannin supplementation caused changes in the composition of the gut microbiota and specifically modulated the populations of butyrate-producing bacteria. The major limitation of this work is that the animals involved in the trial did not show any clear health problems, so we could not evaluate the tannins' potential capability to prevent diseases associated with weaning. The next step could therefore be testing the tannin mixture in artificially infected piglets.

Supporting information

S1 Table. Ingredients and chemical composition of experimental diets.
(DOCX)

S2 Table. Differentially abundant families between the control and tannin groups of piglets. Abbreviations: FDR = false discovery rate <0.05; FC = Fold change. Positive log2 fold change is the relative abundance in the tannin group compared with the control group.
(DOCX)

S3 Table. Differentially abundant genera between the tannin and control groups of animals. Abbreviations: FDR = false discovery rate <0.05. FC = Fold change.

Positive log2 fold fold change is the relative abundance in the tannin group compared with the control group.
(DOCX)

S4 Table. Significant different orthologous groups of proteins between the tannin and control groups. Abbreviations: FDR = false discovery rate <0.05. FC = Fold change. Positive log2 fold change is the relative abundance in the tannin group compared with the control group.
(DOCX)

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Writing – original draft: Francesco Miragoli, Vania Patrone, Alice Senizza, Maria Luisa Callegari.
Writing – review & editing: Vania Patrone, Aldo Prandini, Samantha Sigolo, Luciana Rossi, Alice Senizza.

References

1. Fraga-Corral M, Garcı´a-Oliveira P, Pereira AG, Lourenc¸o-Lopes C, Jimenez-Lopez C, Prieto MA, et al. Technological application of tannin-based extracts. Molecules. 2020; 25: 1–27. https://doi.org/10.3390/molecules25030614 PMID: 32019231
2. Huang Q, Liu X, Zhao G, Hu T, Wang Y. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. Anim Nutri. 2018; 4: 137–150. https://doi.org/10.1016/j.aninu.2017.09.004 PMID: 30140753
3. Marzoni M, Castillo A, Franzoni A, Nery J, Fortina R, Romboli I, et al. Effects of dietary quebracho tannin on performance traits and parasite load in an italian slow-growing chicken (White livorno breed). Animals. 2020; 10: 1–11. https://doi.org/10.3390/ani10040684.
4. Mancini S, Minieri S, Buccioni A, Marzoni Fecia di Cossato M, Russo C, Paci G. The influence of dietary chestnut and quebracho tannins mix on rabbit meat quality. Anim Sci J. 2019; 90: 680–689. https://doi.org/10.1111/asj.13194 PMID: 30848035
5. Aguerre MJ, Duval B, Powell JM, Vadas PA, Wattiaux MA. Effects of feeding a quebracho–chestnut tannin extract on lactating cow performance and nitrogen

utilization efficiency. J Dairy Sci. 2020; 103:2264–2271. https://doi.org/10.3168/jds.2019-17442 PMID: 31864747

6. Ammar H, Lo´pez S, Kammoun M, Bodas R, Gira´ldez FJ, Gonza´lez JS. Feeding quebracho tannins to sheep enhances rumen fermentative activity to degrade browse shrubs. Anim Feed Sci Technol. 2009; 149: 1–15. https://doi.org/10.1016/j.anifeedsci.2008.04.008.
7. Ncube B, Van Staden J. Tilting Plant Metabolism for Improved Metabolite Biosynthesis and Enhanced Human Benefit. Molecules. 2015; 20: 12698–12731. https://doi.org/10.3390/molecules200712698 PMID: 26184148
8. Butler LG. Antinutritional effects of condensed and hydrolyzable tannins. Basic Life Sci. 1992; 59: 693–698. https://doi.org/10.1007/978-1-4615-3476-1_40 PMID: 1417695
9. Bhat TK, Kannan A, Singh B, Sharma OP. Value Addition of Feed and Fodder by Alleviating the Antinutritional Effects of Tannins. Agric Res. 2013; 2: 189–206, https://doi.org/10.1007/s40003-013-0066-6.
10. Biagi G, Cipollini I, Paulicks BR, Roth FX. Effect of tannins on growth performance and intestinal ecosystem in weaned piglets. Arch Anim Nutr. 2010; 64: 121–135. https://doi.org/10.1080/17450390903461584 PMID: 20481351
11. Galassi G, Mason F, Rapetti L, Crovetto GM, Spanghero M. Digestibility and metabolic utilisation of diets containing chestnut tannins and their effects on growth and slaughter traits of heavy pigs. Ital J Anim Sci. 2019; 18: 746–753. https://doi.org/10.1080/1828051X.2019.1570361.
12. Girard M, Bee G. Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. Animal. 2020; 14: 95–107. https://doi.org/10.1017/S1751731119002143 PMID:31571564
13. Wong SYY, Grant IR, Friedman M, Elliott CT, Situ C. Antibacterial activities of naturally occurring compounds against *Mycobacterium avium* subsp. *paratuberculosis*. Appl Environ Microbiol. 2008; 74:5986–5990. https://doi.org/10.1128/AEM.00981-08 PMID: 18676709
14. Burt SA, Van Der Zee R, Koets AP, De Graaff AM, Van Knapen F, Gaastra W, et al. Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7. Appl Environ Microbiol. 2007; 73: 4484–4490. https://doi.org/10.1128/AEM.00340-07 PMID: 17526792
15. Jo´csa´k I, Tossenberger J, Ve´gva´ ri G, Suda´ r G, Varga-VisiE´ , To´ th T. How is the effect of phytogenic feed supplementation tested in heat stressed pigs? Methodological and sampling considerations. Agric. 2020; 10: 1–14. https://doi.org/10.3390/agriculture10070257.
16. Reggi S, Giromini C, Dell'Anno M, Baldi A, Rebucci R, Rossi L. *In Vitro* Digestion of Chestnut and Quebracho Tannin Extracts: Antimicrobial Effect, Antioxidant Capacity and Cytomodulatory Activity in Swine Intestinal IPEC-J2 Cells. Animals. 2020; 10: 195. https://doi.org/10.3390/ani10020195 PMID:31979207
17. Gao J, Yin J, Xu K, Li T, Yin Y. What Is the Impact of Diet on Nutritional Diarrhea Associated with Gut Microbiota in Weaning Piglets: A System Review. Biomed Res Int. 2019; 2019:6916189. https://doi.org/10.1155/2019/6916189 PMID: 31976326
18. Sørensen MT, Vestergaard EM, Jensen SK, Lauridsen C, Højsgaard S. Performance and diarrhoea in piglets following weaning at seven weeks of age: Challenge with *E. coli* O 149 and effect of dietary factors. Livest Sci. 2009; 123: 314–321. https://doi.org/10.1016/j.livsci.2008.12.001.

19. Sun Y, Kim SW. Intestinal challenge with enterotoxigenic *Escherichia coli* in pigs, and nutritional intervention to prevent postweaning diarrhea. Anim Nutr. 2017; 3:322–330. https://doi.org/10.1016/j.aninu.2017.10.001 PMID: 29767133

20. Ruiz VLA, Bersano JG, Carvalho AF, Catroxo MHB, Chiebao DP, Gregori F, et al. Case-control study of pathogens involved in piglet diarrhea. BMC Res Notes. 2016; 9: 22. https://doi.org/10.1186/s13104-015-1751-2 PMID: 26754836

21. Girard M, Thanner S, Pradervand N, Hu D, Ollagnier C, Bee G. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental ETEC F4 model. PLoS One. 2018; 13:e0197878. https://doi.org/10.1371/journal.pone.0197878 PMID: 29799865

22. Liu H, Hu J, Mahfuz S, Piao X. Effects of hydrolysable tannins as zinc oxide substitutes on antioxidant status, immune function, intestinal morphology, and digestive enzyme activities in weaned piglets. Animals. 2020; 10: 757. https://doi.org/10.3390/ani10050757.

23. Choy YY, Quifer-Rada P, Holstege DM, Frese SA, Calvert CC, Mills DA, et al. Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins. Food Funct. 2014; 5: 2298–2308. https://doi.org/10.1039/c4fo00325j PMID: 25066634

24. Vacca M, Celano G, Calabrese FM, Portincasa P, Gobbetti M, De Angelis M. The controversial role of human gut Lachnospiraceae. Microorganisms. 2020; 8: 1–25. https://doi.org/10.3390/microorganisms8040573 PMID: 32326636

25. Vital M, Howe AC, Tiedje JM. Revealing the bacterial butyrate synthesis pathways by analyzing (meta) genomic data. mBio. 2014; 5: e00889. https://doi.org/10.1128/mBio.00889-14 PMID: 24757212

26. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. Microbiome. 2019; 7: 1–15.

27. Zhong X, Zhang Z, Wang S, Cao L, Zhou L, Sun A, et al. Microbial-Driven Butyrate Regulates Jejunal Homeostasis in Piglets During the Weaning Stage. Front Microbiol. 2019; 9: 3335. https://doi.org/10.3389/fmicb.2018.03335 PMID: 30713531

28. Rivera-Cha´vez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, et al. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. Cell Host Microbe. 2016; 19: 443–454. https://doi.org/10.1016/j.chom.2016.03.004 PMID: 27078066

29. Caprarulo V, Hejna M, Giromini C, Liu Y, Dell'Anno M, Sotira S, et al. Evaluation of Dietary Administration of Chestnut and Quebracho Tannins on Growth, Serum Metabolites and Fecal Parameters of Weaned Piglets. Animals. 2020; 10: 1945. https://doi.org/10.3390/ani10111945.

30. Mpendulo CT, Chimonyo M, Ndou SP, Bakare AG. Fiber source and inclusion level affects characteristics of excreta from growing pigs. Asian-Australasian J Anim Sci. 2018; 31: 755–762. https://doi.org/10.5713/ajas.14.0611 PMID: 26954189

31. Council NR. Nutrient Requirements of Swine. Nutrient Requirements of Swine. National Academies Press; 2012. https://doi.org/10.17226/13298.

32. Patrone V, Minuti A, Lizier M, Miragoli F, Lucchini F, Trevisi E, et al. Differential effects of coconut versus soy oil on gut microbiota composition and predicted metabolic function in adult mice. BMC Genomics. 2018; 19. https://doi.org/10.1186/s12864-018-5202-z PMID: 30404613

33. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

34. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25: 2078–2079. https://doi.org/10.1093/bioinformatics/btp352 PMID: 19505943

35. **35.** Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc. 2020; 15: 799–821. https://doi.org/10.1038/s41596-019-0264-1 PMID: 31942082

36. **36.** Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 2017; 45:W180–W188. https://doi.org/10.1093/nar/gkx295 PMID: 28449106

37. **37.** Kurtz ZD, Mu¨ ller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA. Sparse and Compositionally Robust Inference of Microbial Ecological Networks. PLOS Comput Biol. 2015; 11: e1004226. https://doi.org/10.1371/journal.pcbi.1004226 PMID: 25950956

38. Epskamp S, Cramer AOJ, Waldorp LJ, Schmittmann VD, Borsboom D. Qgraph: Network visualizations of relationships in psychometric data. J Stat Softw. 2012; 48: 1–18. https://doi.org/10.18637/jss.v048.i04.

39. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2020. https://www.R-project.org/.

40. Ives AR, Carpenter SR. Stability and diversity of ecosystems. Science. 2007; 317: 58–62. https://doi.org/10.1126/science.1133258 PMID: 17615333

41. Dı´az Carrasco JM, Redondo EA, Pin Viso ND, Redondo LM, Farber MD, Ferna´ndez Miyakawa ME. Tannins and Bacitracin Differentially Modulate Gut Microbiota of Broiler Chickens. Biomed Res Int. 2018; 2018:1879168. https://doi.org/10.1155/2018/1879168 PMID: 29682522

42. Oh JK, Chae JP, Pajarillo EAB, Kim SH, Kwak M, Eun J, et al. Association between the body weight of growing pigs and the functional capacity of their gut microbiota. Anim Sci J. 2020; 91: e13418. https://doi.org/10.1111/asj.13418 PMID: 32648357

43. Mach N, Berri M, Estelle´ J, Levenez F, Lemonnier G, Denis C, et al. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ Microbiol Rep. 2015; 7: 554–569.

44. Kiros TG, Derakhshani H, Pinloche E, D'Inca R, Marshall J, Auclair E, et al. Effect of live yeast *Saccharomyces cerevisiae* (Actisaf Sc 47) supplementation on the performance and hindgut microbiota composition of weanling pigs. Sci Rep. 2018; 8: 1–13.

45. Yang H, Huang X, Fang S, He M, Zhao Y, Wu Z, et al. Unraveling the Fecal Microbiota and Metagenomic Functional Capacity Associated with Feed Efficiency in Pigs. Front Microbiol. 2017; 8: 1555. https://doi.org/10.3389/fmicb.2017.01555 PMID: 28861066

46. Yang Q, Huang X, Wang P, Yan Z, Sun W, Zhao S, et al. Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. Microbiologyopen. 2019; 8 (12): e923. https://doi.org/10.1002/mbo3.923 PMID: 31496126

47. McCormack UM, Curião T, Buzoianu SG, Prieto ML, Ryan T, Varley P, et al. Exploring a possible link between the intestinal microbiota and feed efficiency in

pigs. Appl Environ Microbiol. 2017; 83. https://doi.org/10.1128/AEM.00380-17 PMID: 28526795

48. Tigchelaar EF, Bonder MJ, Jankipersadsing SA, Fu J, Wijmenga C, Zhernakova A. Gut microbiota composition associated with stool consistency. Gut. BMJ Publishing Group; 2016. pp. 540–542. https://doi.org/10.1136/gutjnl-2015-310328 PMID: 26276682
49. Gophna U, Konikoff T, Nielsen HB. Oscillospira and related bacteria—From metagenomic species to metabolic features. Environ Microbiol. 2017; 19: 835–841. https://doi.org/10.1111/1462-2920.13658 PMID: 28028921
50. Schokker D, Zhang J, Zhang LL, Vastenhouw SA, Heilig HGHJ, Smidt H, et al. Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. PLoS One. 2014; 9(6):e100040. https://doi.org/10.1371/journal.pone.0100040 PMID: 24941112
51. Million M, Tomas J, Wagner C, Lelouard H, Raoult D, Gorvel JP. New insights in gut microbiota and mucosal immunity of the small intestine. Hum Microbiome J. 2018; 7: 23–32. https://doi.org/10.1016/j.humic.2018.01.004.
52. Holmes AJ, Chew YV, Colakoglu F, Cliff JB, Klaassens E, Read MN, et al. Diet-Microbiome Interactions in Health Are Controlled by Intestinal Nitrogen Source Constraints. Cell Metab. 2017; 25: 140–151. https://doi.org/10.1016/j.cmet.2016.10.021 PMID: 27889387
53. Fernandes J, Su W, Rahat-Rozenbloom S, Wolever TMS, Comelli EM. Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. Nutr Diabetes. 2014; 4: e121. https://doi.org/10.1038/nutd.2014.23 PMID: 24979150
54. Lærke HN, Jensen BB. D-tagatose has low small intestinal digestibility but high large intestinal fermentability in pigs. J Nutr. 1999; 129: 1002–1009. https://doi.org/10.1093/jn/129.5.1002 PMID: 10222392
55. Duncan SH, Louis P, Flint HJ. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol. 2004; 70: 5810–5817. https://doi.org/10.1128/AEM.70.10.5810-5817.2004 PMID: 15466518
56. Newman MA, Petri RM, Gru¨ ll D, Zebeli Q, Metzler-Zebeli BU. Transglycosylated starch modulates the gut microbiome and expression of genes related to lipid synthesis in liver and adipose tissue of pigs. Front Microbiol. 2018; 9. https://doi.org/10.3389/fmicb.2018.00224 PMID: 29487593
57. Heinbockel L, Weindl G, Martinez-de-Tejada G, Correa W, Sanchez-Gomez S, Ba´rcena-Varela S, et al. Inhibition of Lipopolysaccharide- and Lipoprotein-Induced Inflammation by Antitoxin Peptide Pep19-2.5. Front Immunol. 2018; 9: 1704. https://doi.org/10.3389/fimmu.2018.01704 PMID: 30093904
58. d'Hennezel E, Abubucker S, Murphy LO, Cullen TW. Total Lipopolysaccharide from the Human Gut Microbiome Silences Toll-Like Receptor Signaling. mSystems. 2017; 2(6):e00046–17. https://doi.org/10.1128/mSystems.00046-17 PMID: 29152585
59. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front Microbiol. 2016; 7: 979. https://doi.org/10.3389/fmicb.2016.00979 PMID: 27446020
60. Kopečny´ J, Zorec M, Mra´zek J, Kobayashi Y, Marinsˇek-Logar R. *Butyrivibrio hungatei* sp. nov. and *Pseudobutyrivibrio xylanivorans* sp. nov., butyrate-producing bacteria from the rumen. Int J Syst Evol Microbiol. 2003; 53: 201–209. https://doi.org/10.1099/ijs.0.02345-0 PMID: 12656174

61. Deutscher J, Francke C, Postma PW. How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. Microbiol Mol Biol Rev. 2006; 70: 939–1031. https://doi.org/10.1128/MMBR.00024-06 PMID: 17158705

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Implications of Tributyrin on Gut Microbiota Shifts Related to Performances of Weaning Piglets

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Brief Introduction to the Study

After interesting results obtained from the *in vivo* trial where tributyrin enhanced piglets growth, this study importantly contributed to the scientific knowledge of gut microbiota modulation after tributyrin supplementation. The dietary supplementation of 0.2% of tributyrin strongly modulated the gut microbiota of piglets increasing the beta diversity. This paper was the first study which assessed the effect of tributyrin on gut microbiota in post-weaning piglets. Obtained findings showed that tributyrin promoted the abundance of several genera that are positively correlated to animal performance and increased the isobutyrate concentration in faeces suggesting higher protein utilization.

5.2 Implications of Tributyrin on Gut Microbiota Shifts Related to Performances of Weaning Piglets

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Abstract: Alternatives to antibiotic treatments are required owing to the ban on the use of these drugs as growth promoters in food animal production. Tributyrin appears to play a role in improving growth performance in pigs, albeit with varying degrees of effectiveness. So far, very little is known about its effects on gut microbiota composition. In this study, we investigated the gut microbiota changes of piglets

receiving, at weaning, 0.2% tributyrin added to their basal diet. Microbiota composition was assessed through 16S-rRNA gene sequencing on stools collected from tributyrin and control groups. The functional profiles of microbial communities were predicted from amplicon abundance data. A comparison between dietary groups revealed that tributyrin strongly modulated gut microbiota composition in piglets, increasing the relative abundance of a number of bacterial genera such as *Oscillospira*, *Oscillibacter*, *Mucispirillum* and *Butyrivibrio*. These genera were positively correlated to animal average daily gain (ADG) and/or body weight (BW). Based on the function profile prediction, the gut microbiome of the tributyrin group possessed an enhanced potential for energy metabolism and a reduced potential for carbohydrate metabolism. In conclusion, our results indicated that tributyrin can promote changes to gut microbial communities, which could contribute to improving animal performance after weaning.

Keywords: weaning; piglets; gut microbiota; tributyrin; animal performance

1. Introduction

Early weaning represents a practice that offers economic benefits for farmers, and for this reason, it is adopted by most intensive breeding practitioners in developed countries. Weaning is a period in the life of piglets characterized by several stressing factors that lead to reduced performance and contribute to the development of intestinal dysfunctions such as postweaning diarrhea. It is well known that, during weaning, several factors shape the gut microbiota composition [1]. Among these, changes of diet represent one of the most impactful conditions [2] and can be related to diarrhea. Other factors, such as infectious agents [3–5] and the weaning-mediated evolution of the gut microbiota [6–8] are also responsible for the development of these intestinal diseases.

Antibiotic treatments still represent the only strategy for controlling diseases related to weaning, though a series of feed supplements have been proposed as effective alternatives to drug treatments [9–11] following the ban of food animal growth-promoting antibiotics by the European Union in 2003 (EC No 1831/2003) [12]. In this context, tributyrate glyceride, also called tributyrin, appears to be a good candidate for alleviating intestinal dysfunctions, modulating immune response and improving growth performance in piglets. Despite the large number of papers addressing tributyrin impact on weaning piglets, its efficacy as a growth promoter is sometimes controversial [13–16] probably because of different experimental conditions in farm trials. Indeed, the efficiency of tributyrin, as with all other growth

promoter candidates, may vary based on its mechanisms of action, the basal diet composition and the animal's health conditions. Despite several studies concerning butyric acid and its derivatives, their mechanisms of action are still unclear. Morphological changes of the intestinal mucosa are one of the impacting factors on the animal's welfare, and some studies indicated that tributyrin could alleviate these weaning-related symptoms. In particular, tributyrin demonstrated effectiveness in modifying crypt depth and villus height in different intestinal sections [13,15–18].

Thus, all these results suggest that tributyrin supplementation, albeit with varying degrees of effectiveness, plays a role in alleviating the detrimental effects on gastrointestinal barrier properties caused by early weaning that can lead to inflammatory status. Moreover, Gu et al. [19] have reported an anti-inflammatory action of tributyrin in piglets as a consequence of the intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS), a model of enteropathogenic infections. Another proposed mechanism of tributyrin action is the reduction in reactive oxygen species (ROS) [20] and the protection of mitochondrial function against oxidative stress [17].

Antimicrobial activities of organic acids and their derivatives, including monobutyrin, have been reported in an *in vitro* study [21]. The antimicrobial effects of butyrate glycerides on *Salmonella Typhimurium* and *Clostridium perfringens* were studied in poultry [22]. However, there is little information regarding the effects of tributyrin on pigs' intestinal microbiota, with most of the studies being concerned with bacteria considered as an indicator of animal health [14,16]. In our previous study [14], we identified significant differences in body weight (BW), average daily gain (ADG) and several blood parameters in piglets receiving tributyrin during the weaning period, as compared to the control. In that study, we specifically addressed the impact of tributyrin on lactobacilli and bifidobacterial, although other gut bacterial populations have been associated with high feed efficiency in pigs [23,24]. The aim of the present work was thereby to comprehensively assess the gut microbiota composition of pigs fed tributyrin by means of high-throughput 16S-rRNA gene sequencing. In the present work, the fecal samples from the aforementioned animal trial were exploited to gain insight into the structure and metabolic potential of gut microbial communities, along with the production of short-chain fatty acids (SCFAs). Microbiological data were correlated with physiological and performance parameters measured in the previously mentioned work, in order to investigate how changes to gut microbiota could impact both animal health and performance.

2. Materials and Methods

2.1 Animals and Sample Collection

The animals used in the present study were selected among those involved in the trial described by Sotira et al. [14]. For the full details concerning animal experimentation and ethics, basal diet composition, breeding type and experimental design, we refer the reader to this previous publication. The trial was approved by the animal welfare body of University of Milan (authorization number 31/2019) according to Italian regulation on animal experimentation and ethics (DL 26/2014) and to European regulation (Dir. 2010/63). All procedures were carried out according to relevant guidelines and regulations. Briefly, 120 weaned piglets (28 ± 2 days) were allotted in a randomized complete block design into two experimental groups: control (CTR) and tributyrin (TRI). Each group constituted 60 pigs (6 replicate pens with 10 pigs per pen). The groups were homogeneous for gender, weight and litter. Furthermore, the two groups were raised in the same room with a free floor surface where temperature (27 °C) and humidity (60%) were controlled. Water and feed were supplied *ad libitum*. The area available for each animal was 0.40 m^2, according to the Directive 2008/120/EC. After one-week of adaptation in which the animals received the basal diet (corresponding to the day 0 and 35 days from birth), experimental diets were fed ad libitum for each group for 40 days. The CTR group received the basal diet whereas the TRI group received the same basal diet supplemented with 0.2% of tributyrin (ACIFIS® Tri-B, New Feed Team srl, Lodi, Italy). The experimental diets were isoenergetic and isoproteic and complied with the NRC requirements [25] for postweaned piglets, as already reported by Sotira et al. [14] (Table S1). Two animals per pen were then randomly selected for each treatment group for faecal sample collection. At day 40, corresponding to 75 days from the birth of piglets, stool samples were individually collected from rectal ampulla and immediately frozen in dry ice. Once transferred to the laboratories, they were stored frozen at ⊚80 _C until DNA extraction.

2.2 DNA Extraction, 16S rRNA Gene Amplification and Sequencing

Total bacterial DNA was extracted from 50mg (wet weight) using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany), following the manufacturer's instructions. The genomic DNA was then quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA), while the quality of the extracted DNA was assessed using agarose gel electrophoresis. DNA amplification was performed using primers 343F and 802R, which allowed the amplification of the V3–V4 regions of the bacterial 16S rRNA gene. Amplification was carried out as

described above [26]. The PCR products were then analyzed using agarose gel electrophoresis and quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA). Finally, a pool of amplicons was prepared by adding the PCR products of each sample in an equimolar concentration and then purified using a DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA).

Sequencing was performed at Fasteris SA (Geneva, Switzerland) using Illumina's MiSeq v3 platform with 2 × 300 bp mode. Raw reads were filtered for low quality (quality score ≥ 30) by Trimmomatic tool version 0.32 (http://www.usadellab.org/cms/index.php?page=trimmomatic, accessed on 26 August 2020) [27] by sliding window trimming (window size: 4 bases, quality: 15). Reads showing a length shorter than 60 bases were excluded. Overlapped reads were mapped against the SILVA database (Version SSURef_NR99_115_tax_silva_DNA.fasta) using Burrows–Wheeler Alignment Tool version 0.7.5a (http://bio-bwa.sourceforge.net/, accessed on 28 August 2020) [28]. The SSU dataset was composed of high-quality 16S/18S rRNA sequences (99% criterion) applied to remove redundant sequences. In order to merge alignments and to compute the number of reads onto each OTU, the SAM tools package was used [28]. Sequence files were deposited in the European Nucleotide Archive (ENA) database under the accession number PRJEB40653.

2.3. Gas-Chromatographic Analysis of Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) were extracted from a 3 g sample dissolved in 9 mL of distillate water by stirring for a few minutes. Following centrifugation at 480_ g for 15 min, 2 mL of supernatant was added to 1 mL of a pivalic acid solution (internal standard, 1 g L^{-1} in distilled water) and 1 mL of a 0.12 M oxalic acid solution. This suspension was mixed using a vortex and then centrifuged at 480_ g. The upper phase was microfiltered.

The gas-chromatographic analysis was carried out using a Shimadzu 2025 gas chromatograph equipped with an AOC-20i autosampler (Shimadzu Srl, Milan, Italy), an FID detector and a 30 m _ 0.250 mm, 0.25 _m DB-FFAP capillary column (Agilent Technologies, Inc. Santa Clara, CA, USA). The injector and detector temperatures were 200 and 220 °C, respectively. The injection was carried out in split mode (1:100), and a volume of 1 μL was injected. The analysis was carried out at a constant flow of hydrogen gas using a programmed temperature: the initial temperature was 60 _C and held for 5 min. The temperature was then raised to 160 °C at 5 °C min^{-1} and finally to 190 °C at 10 °C min^{-1} and held for 7 min.

3. Results

3.1. Microbiota Composition and Community Diversity Associated with Tributyrin Supplementation

The total number of filtered sequences obtained in the samples collected from tributyrin and control animal groups was 2,405,009. The median sequencing coverage was 100,209 sequences per sample with minimal and maximal coverages of 65,975 and 171,531 reads, respectively. Based on principal coordinates analysis (PCoA), we observed significant diet-specific differences in gut bacterial communities (Figure 1), with the supplementation of tributyrin accounting for about 20% of the total variance (Permanova analysis, $R2 = 0.19$, $p < 0.01$). Tributyrin supplementation had no significant influence on fecal bacterial alpha diversity calculated by Chao 1 ($p = 0.195$), observed OTUs ($p = 0.102$), Simpson ($p = 0.737$) and Shannon ($p = 0.144$) indices. These results indicated that no significant differences were observed in term of species diversity and richness among dietary groups. At the phylum level, the fecal microbiota collected from the control group was dominated by *Firmicutes* (77.97%) followed by *Actinobacteria* (13%) and *Bacteroidetes* (6.52%), whereas animals receiving tributyrin supplementation showed a microbiota dominated by *Firmicutes* (73%) followed by *Bacteroidetes* (15%) and *Actinobacteria* (1%) (Figure 2A). In this group, the relative abundance of Firmicutes and Actinobacteria decreased significantly in fecal samples (FRD = 0.002 and FDR = 0.0235, respectively), while a significant increase was detected in the levels of *Proteobacteria* (FDR = 0.0150). Additionally, the relative abundance of *Cyanobacteria*, *Deferribacteres* and *Spirochaetae* (Supplementary Table S2) increased in tributyrin-fed piglets.

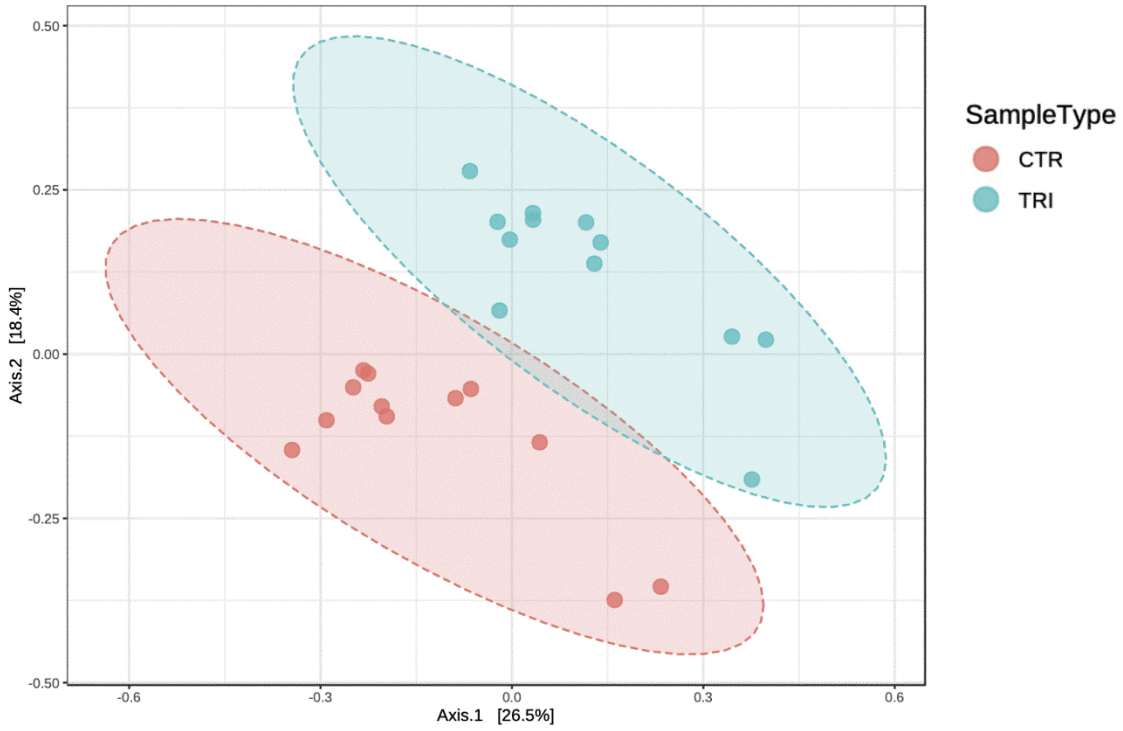

Figure 1. Principal coordinates analysis (PCoA, Bray-Curtis distance) plot of the gut microbiota of weaning piglets fed a diet with (TRI) or without tributyrin supplementation (CTR) ($R2 = 0.19$, $p < 0.01$).

At the family level, *Desulfovibrionaceae*, *Mycoplasmataceae*, *Alcaligenaceae*, *Campylobacteriaceae*, VadinBB60 and *Deferribacteraceae* proved to be significantly enriched in samples collected from the tributyrin group. In addition, the tributyrin supplementation caused significant reductions in the relative abundances of *Coriobacteriaceae*, *Peptococcaceae*, *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae* and Family XIII *Incertae Sedis* (Figure 2B and Supplementary Table S2).

Moreover, at the genus level, significant increases in *Oscillospira*, *Desulfovibrio*, *Pseudoflavonifractor*, *Butyrivibrio*, *Sutterella*, *Oscillibacter*, *Mycoplasma*, *Campylobacter*, *Anaerotruncus*, *Mucispirillum* and the RC9 gut group genera was found in the tributyrin group samples. In addition, *Mogibacterium*, *Collinsella*, *Peptococcus*, *Atopobium*, *Subdoligranulum*, an uncultured bacterium, *Syntrophococcus*, *Marvinbryantia*, *Blautia*, *Enterorhabdus*, *Oribacterium* and *Denitrobacterium* genera levels were significant reduced following tributyrin supplementation (Figure 2C and Supplementary Table S3).

Moreover, Random Forest analysis revealed that at the family level, *Coriobacteriaceae* and Family XIII *Incertae Sedis* were the most important features in discriminating between the two groups of animals. Among the members of the aforementioned families, the genera *Mogibacterium*, *Collinsella*, *Peptococcus* and

Atopobium were the most important discriminant genera, since their representative points are on the right and at the top of the graph, indicating their higher predictive values (Figure 3).

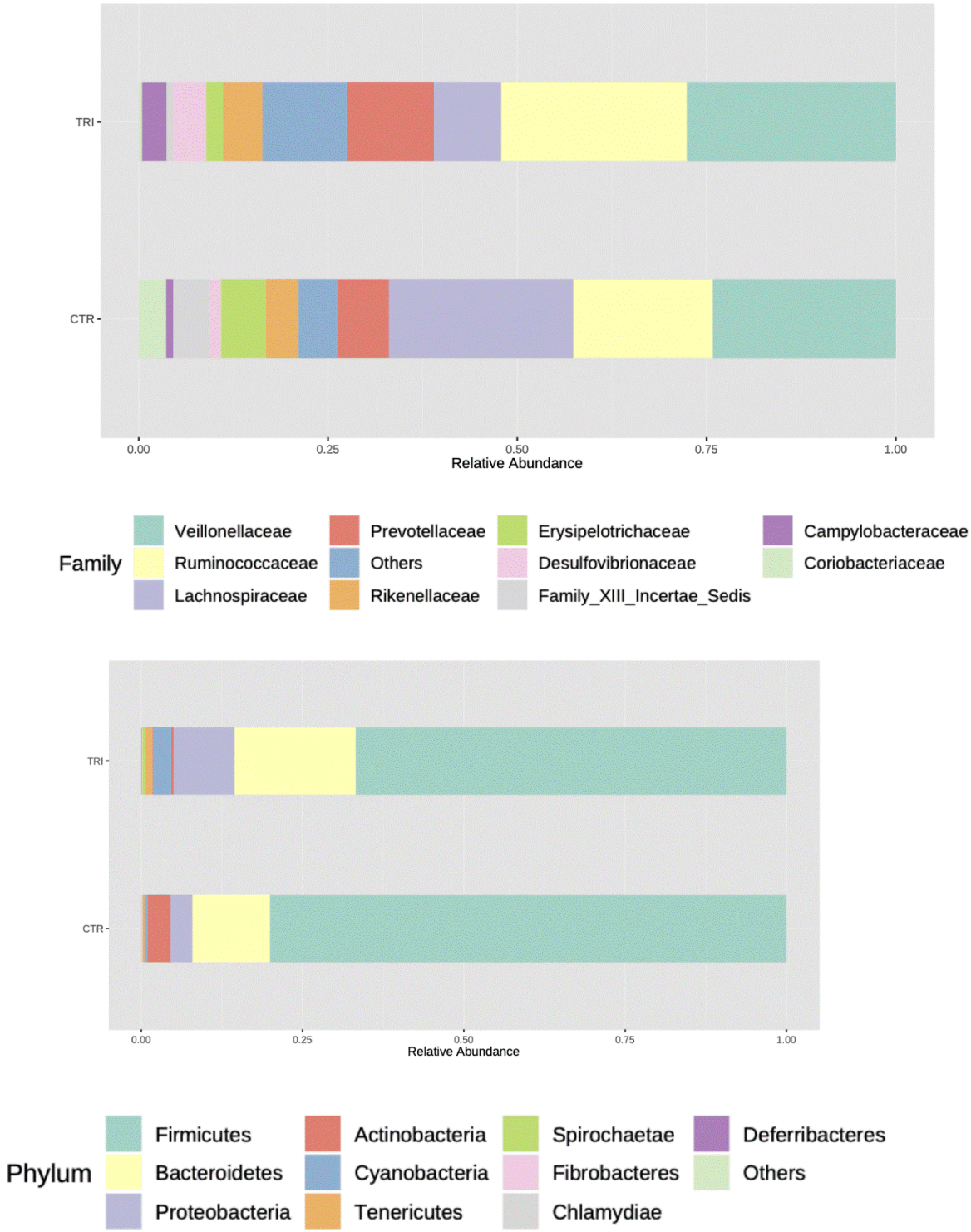

Figure 2. Cont.

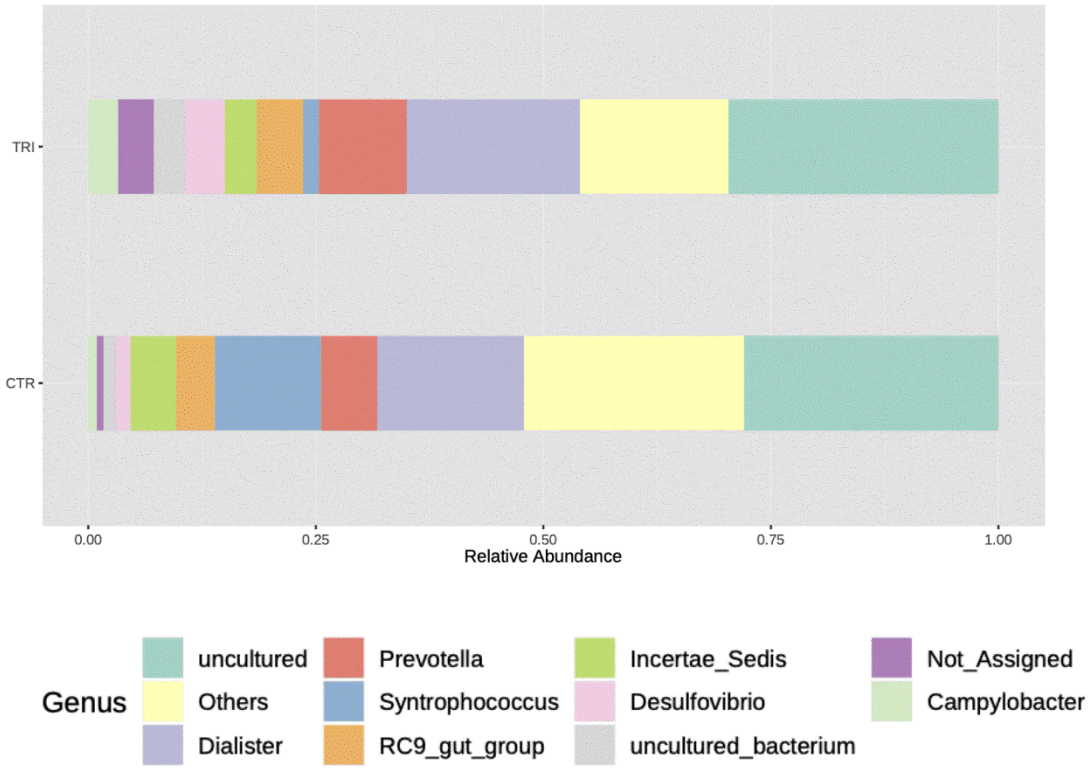

Figure 2. Relative abundances of the different phyla (**A**), families (**B**) and general (**C**) observed in tributyrin animal group (TRI) compared with those of the control group (CTR). Only top taxa are shown.

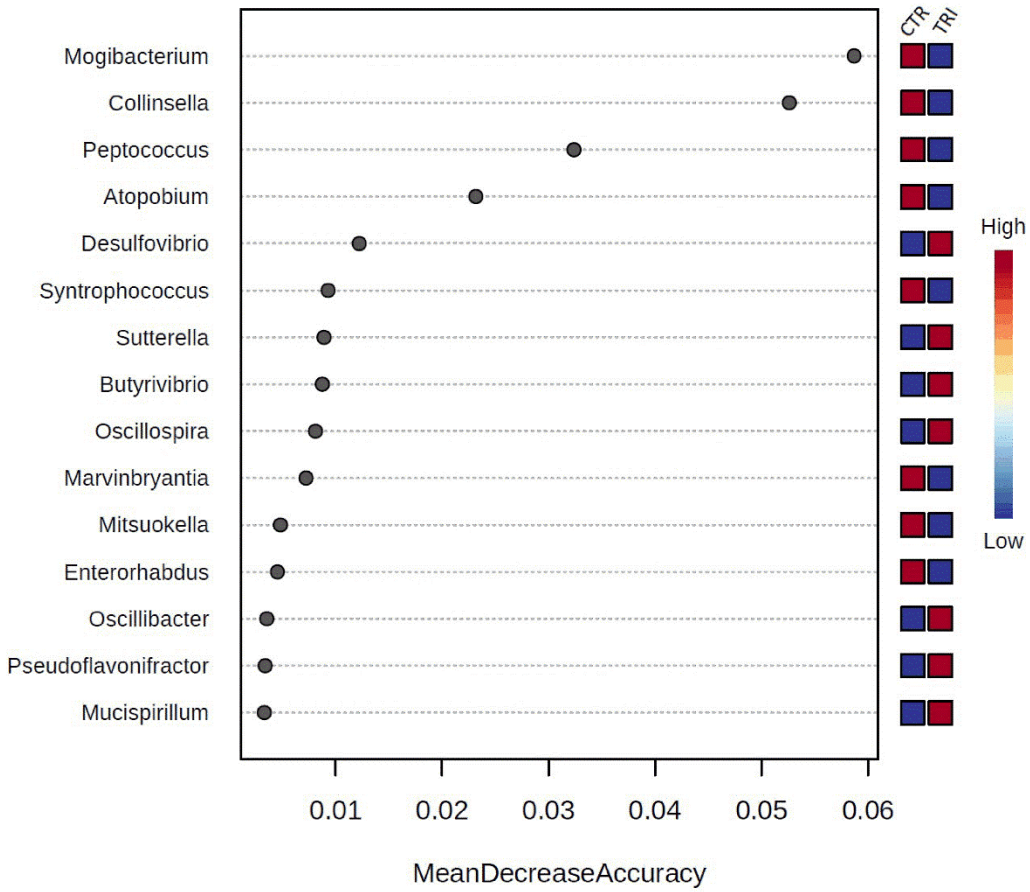

Figure 3. The 15 most discriminant genera between control and tributyrin sample sequences as determined by Random Forest analysis using mean decrease in accuracy.

3.2. Functional Prediction

The operational taxonomic unit (OTU) table obtained through sequence analysis was uploaded to the MicrobiomeAnalyst software using the Marker Data Profiling option. The Kegg Onthology assignment table was generated using Tax4Fun and was then used for further analysis via the Shotgun data profiling option provided by the same software. Random Forest analysis revealed that the most discriminant COG functional categories between the two groups of samples were carbohydrate and nucleotide metabolisms, more prominently represented in the control group, whereas energy and amino acid metabolisms were enriched in the tributyrin group (Figure 4). Based on EdgeR analysis, only the energy metabolism pathways were found to be significantly increased (FDR = 0.007) in the tributyrin group, combined with a significant reduction in carbohydrate metabolism (FDR = 0.007). In particular, in the control group, we found enriched genes encoding sugar transport, mainly components of phosphotransferase systems (K02768, K02769, K02793, K02795 and K02796). Conversely, in the tributyrin samples, genes encoding amino acid utilization as an energy source (K00830, K00830 and K02204) were enriched, compared to the control

group. Other genes were identified as being more prominently represented in the samples of the tributyrin group, some of which were related to gluconeogenesis (K03841 and K16153) and others to the glyoxylate cycle (K00122, K01638, K00283 and K00605).

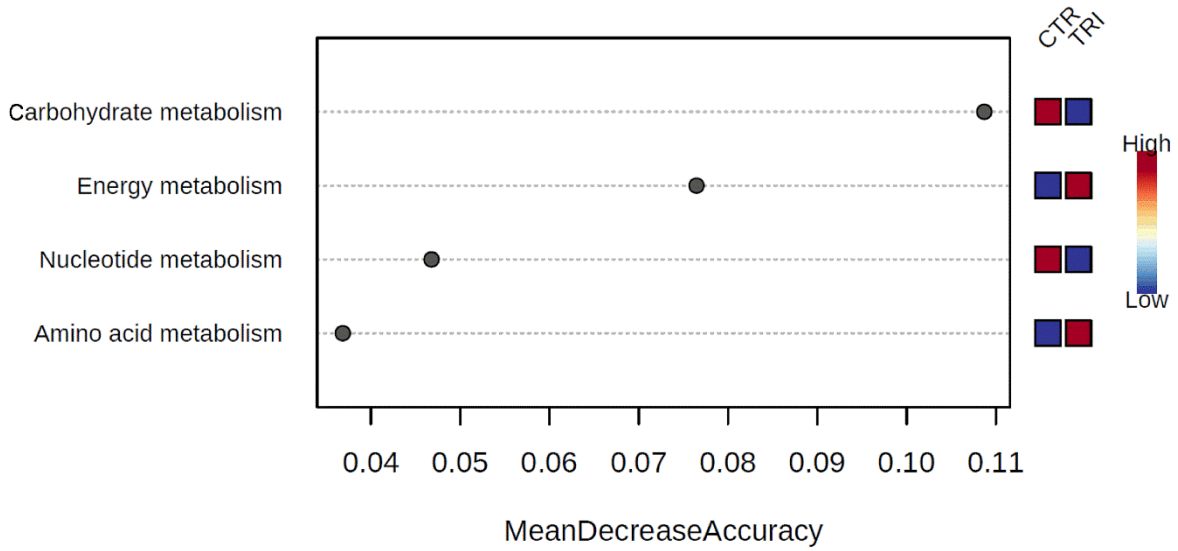

Figure 4. The most discriminant Cluster of Orthologous Groups (COG) functional categories between control and tributyrin samples determined by Random Forest analysis using mean decrease in accuracy.

3.3. SCFA Concentration in Stools

The SCFA concentration in the stools of both dietary groups of animals was recorded. The most abundant SCFAs were acetate, propionate and butyrate, which did not reveal significant differences between the two animal groups, along with isovalerate and valerate. However, the tributyrin group of piglets revealed a 1.31-fold increase in their fecal concentration of isobutyrate, and this difference between the two groups of animals was statistically significant ($p = 0.0269$) (Table 1).

Table 1. Mean values of short-chain fatty acid (SCFA) concentrations of tributyrin (TRI) and control (CTR) piglet samples. Data are presented as mmol% ± SD.

	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
CTR (N= 12)	58.63±6.15	23.93±4.45	1.74±0.91	9.93±2.41	2.25±1.37	3.51±1.09
TRI (N= 12)	57.22±3.35	22.72±2.64	2.29±0.82	10.84±1.68	3.00±1.24	3.93±0.95
p-value	0.8852	0.2983	0.0269	0.4357	0.1410	0.5067

3.4. Correlation between Microbiota and Piglet Physiological/Performance Parameters

Spearman's correlations were calculated between the physiological and performance data evaluated in our previous work and significant genera were found via statistical analysis. The correlation results are graphically presented in Figure 5, while the detailed data are presented in Supplementary Table S4.

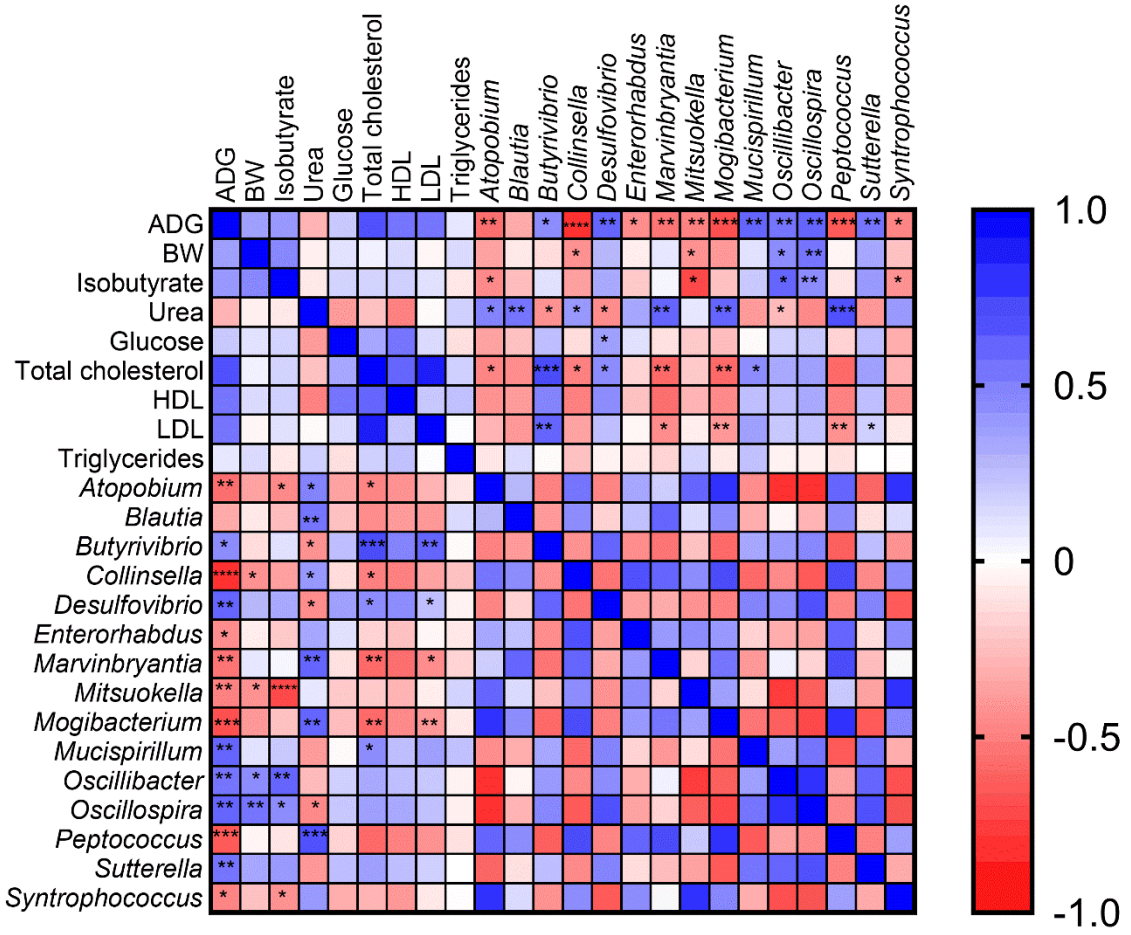

Figure 5. The Spearman correlation heatmap graphically represents the correlation between Average Daily Gain (ADG), Body Weight (BW), isobutyrate, urea, total cholesterol, High-Density Lipoproteins (HDLs), Low-Density Lipoproteins (LDLs) and significant different genera between the two dietary groups. The colors display the *r*-value. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ and **** $p \leq 0.0001$. p value significances were graphically reported only for correlation between the aforementioned parameters and bacterial genus.

Positive correlations were found between *Butyrivibrio* (r = 0.45, p = 0.03), *Desulfovibrio* (r = 0.59, p = 0.002), *Oscillibacter* (r = 0.53, p = 0.007), *Sutterella* (r = 0.54, p = 0.007) and ADG. Conversely, *Atopobium* (r = –0.56, p = 0.005), *Collinsella* (r = –0.81, p = 0.000002), Enterorhabdus (r = –0.44, p = 0.03), Marvinbryantia (r = –0.53, p = 0.007), Mitsuokella (r = –0.49, p = 0.015), *Mogibacterium* (r = –0.68, p = 0.0003), *Peptococcus* (r = –0.65, p = 0.0006) and *Syntrophococcus* (r = –0.47, p = 0.019) revealed negative correlations within the aforementioned animal parameters. Moreover, *Oscillibacter* (r = 0.44, p = 0.03) and *Oscillospira* (r = 0.55, p = 0.0056) were also positively correlated with animal BW. Interestingly, a positive correlation was found between isobutyrate and both *Oscillibacter* (r = 0.57; p = 0.003607) and *Oscillospira* (r = 0.60, p = 0.0017), whereas Atopobium (r = –0.45; p = 0.026), *Collinsella* (r = –0.37; p = 0.07), *Mitsuokella* (r = –0.71; p = 0.00009) and *Syntrophococcus* (r = –0.42; p = 0.043) were negatively correlated.

Atopobium ($r = 0.48$; $p = 0.017$), *Blautia* ($r = 0.53$; $p = 0.007$), *Collinsella* ($r = 0.42$; $p = 0.043$), *Marvinbryantia* ($r = 0.60$; $p = 0.0020$), *Mogibacterium* ($r = 0.62$; $p = 0.0013$), and *Peptococcus* ($r = 0.67$; $p = 0.0003$) were positively correlated with urea concentration values. Conversely, negative correlations were found between urea and *Butyrivibrio* ($r = -0.43$; $p = 0.037$) and *Desulfovibrio* ($r = -0.44$; $p = 0.032$).

4. Discussion

The primary aim of this work was to investigate the effects of tributyrin on the gut microbiota of weaned piglets. Secondly, we focused our attention on how the composition of these communities could be associated with animal performances. Using 16S rRNA gene sequencing, we detected a number of compositional changes of fecal bacterial populations indicating that tributyrin substantially altered the gut microbial community of piglets. Actinobacteria was the most significantly decreased phylum in tributyrin-fed animals, driven by a significant reduction in the family *Coriobacteriaceae* and the genera *Collinsella* and *Atopobium*. In addition, Random Forest analysis revealed that the most discriminant genera between the two dietary groups of animals were *Mogibacterium, Collinsella, Peptococcus*, and *Atopobium*. All these genera decreased in the tributyrin group compared with controls. Little information regarding these genera is available. *Collinsella* is known to establish persistent colonization of intestinal mucosa via utilization of mucins in both pigs and humans [31–33]; its close association with the mucus layer suggests a direct interaction between these microorganisms and the host's intestinal tissues. A decrease in the relative abundance of *Collinsella* has been previously described in several studies [34–36] as being associated to reduced gut permeability and hence to an improved functionality of the intestinal barrier. *Mogibacterium*, a genus belonging to the *Clostridium Family XIII Incertae Sedis,* has been shown to increase in mucosa-associated microbiota of colon cancer patients [37] but to decrease, together with *Collinsella*, in the feces of neonatal pigs administered with a beneficial prebiotic preparation [38]. Burrough et al. [39] reported a high relative abundance of *Mogibacterium* in both mucosal scrapings and luminal samples from pigs with swine dysentery. *Mogibacterium* spp. produce phenyl acetate as their unique final metabolic product [40] and they have been associated with ammonia assimilation in the rumen of cows [41]. Tributyrin supplementation also resulted in lower numbers of *Peptococcus*; this taxon has been often detected in the gut of pigs and its levels have been reported to be negatively associated with feed conversion ratio [42] but positively correlated with preweaned weight gain [43]. Based on our correlation results, *Collinsella* displayed a strong negative correlation with both ADG and BW, suggesting a potential relationship between this population and pig performance. Our results are in line with those of Kubasova et al. [44], who showed

that the microbiota of high-residual feed intake pigs were enriched in *Collinsella* compared with animals that were more efficient. *Mogibacterium, Peptococcus* and *Atopobium* correlated negatively to ADG parameter as well. A positive correlation between *Mogibacterium* and blood urea concentration was also found; from such a result, we can only speculate that the decrease in this genus in tributyrin-fed animals is somewhat linked to the decrease in plasma urea previously described. The higher abundance of Proteobacteria observed in the stool of tributyrin-fed piglets was driven by a significant increase in the *Desulfovibrio* genus. Indeed, this could be related to an increased hydrogen production in the lumen, as the growth of members of this genus is highly dependent on this gas. Since *Desulfovibrio* species are hydrogen consumers, they play a key role in removing this fermentation inhibitor from the intestinal lumen [45]. This important role in the progression of fermentation could explain the positive correlation of *Desulfovibrio* with the ADG parameter we identified. In addition to the above-described populations, other bacterial taxa resulted differentially present in the gut microbiota of tributyrin-fed piglets [46]. Most of the genera that are significantly increased in the tributyrin group have been described in previous works as enriched in high-feed efficiency pigs. In particular, McCormack et al. [23] reported that, in weaning piglets, the *Butyrivibrio* genus was present only in animals showing high performances, which could be due to the enhanced ability of their gut microbiota to ferment complex carbohydrates. In the same study, the *Oscillibacter* genus was associated with improved weight gain [47]. Both *Butyrivibrio* and *Oscillibacter* were the genera that increased mostly significant in the tributyrin group, along with *Mucispirillum*. Likewise, the latter genus showed higher relative abundance in low residual feed intake (RFI) animals [24] and was found to be present uniquely during the postweaning phase. *Mucispirillum* is an oppor tunistic mucin utilizer that has been identified as part of an immunogenic commensal group in humans. This genus adheres to the intestinal mucus and interacts with the host immune system; through a T-dependent IgA response, they never become dominant [48]. This information is reassuring with regard to the role of *Mucispirillum*, since it has sometimes been assumed to be harmful to the integrity of the mucus layer [49]. Our results seem to confirm these positive roles played by *Butyrivibrio* and *Mucispirillum* in animal performances, as indicated by their positive correlations with ADG values. Moreover, *Oscillibacter* positively correlated with BW. As for *Oscillospira*, species belonging to this genus are capable of using alternative energy sources such as mucins and glucoronate [50]; the reduced number of sugar transporter genes in their genomes has been correlated to their inability to use plant glycans. In humans, *Oscillospira* was positively correlated with harder stools [51], and the abundant reduction in this genus was associated with inflammatory status [52]. If confirmed in swine, both of these

properties could be useful during the weaning period to counteract some of the negative effects of solid-diet introduction, such as diarrhea and inflammation. Based on our analysis, *Oscillospira* was positively correlated with both ADG and BW. Other significant differences were detected in the tributyrin group for another genus, *Sutterella*, which could potentially affect gut metabolism. Indeed, Argüello et al. [53] have hypothesized the interfering with the gut colonization of pathogenic Proteobacteria by competition or by stimulation of the immune system. While the role of *Sutterella* is still unclear, the genus has been positively associated with average daily feed intake (ADF) [54]. Our results also confirm a positive correlation of this genus with ADG and ADF, though we did not detect significant differences in feed intake between the two animal groups. Changes in fecal SCFA concentrations can reflect the different balance between groups of bacteria present in the gut microbiota. However, the SCFA concentration in stools must be considered as an approximation of gut microbiota metabolism products since, immediately after their production, enteric cells remove them from the intestinal lumen [55]. In spite of the changes detected in gut microbiota composition due to tributyrin, the determination of SCFA did not reveal significant differences between the two groups of animals, with the exception of a significant increase in isobutyrate. The higher amount of isobutyrate in the tributyrin group could indicate that the supplementation improved diet protein catabolism since this branched short-chain fatty acid is the result of valine degradation [56]. The increase in isobutyrate has been proposed as a parameter of diet protein utilization that characterizes the microbiota of high-feed efficiency pigs [23]. Little is known about the physiological impact of this branched short-chain fatty acid, even though isobutyrate has been shown to be involved in glucose and lipid metabolisms [57]. Interestingly, in our study *Oscillospira* and *Oscillibacter* showed a weak though significant positive correlation with fecal levels of isobutyrate.

Finally, in order to investigate changes in the functional capacity of gut microbiota as a result of dietary intervention, a functional metagenomics prediction approach was adopted. The analysis of the pathways of both animal groups using the KEGG reference pathways allowed us to highlight the reduction in carbohydrate metabolisms in the tributyrin group and, in particular, genes involved in sugar transport such as PTS transport systems. Conversely, in the same group of animals, genes involved in energy metabolism were enriched. The reduction in carbohydrate metabolism and, in particular, the sugar transport systems, seemed to be compensated by other metabolic pathways, such as those of amino acid catabolism, gluconeogenesis and the glyoxylate cycle. Commonly, bacteria use amino acid as an energy source after their deamination or decarboxylation. On the other hand, the increase in isobutyrate production could be ascribed to an increase in amino acid catabolic pathways.

Concerning the enrichment of genes involved in both gluconeogenesis and glyoxylate, it has been suggested that both pathways are used as a response to limited sources of fermentable carbohydrates. The glyoxylate shunt has been investigated in E. coli, Salmonella and other pathogens [58], though it is widespread among bacteria. The entire glyoxylate pathway of *Butyrivibrio proteoclasticus* is available in the KEGG database. Though we found no additional details, we can speculate that other species belonging to the genus *Butyrivibrio* can harbor genes encoding for this pathway as well as other intestinal bacteria. Accordingly, it is very difficult to hypothesize a putative implication of these pathways for the host's health and performances. However, it is important to underline that these prediction analyses are based on genome sequencing data and, therefore, do not provide information concerning gene expression and its regulation. Conversely, tributyrin selected groups of bacteria that harbor genes coding for glyoxylate shunt enzymes. Further analysis is required, and a metabolomics approach could help in evaluating both the reduction in carbohydrate metabolisms and the increase in gluconeogenesis and glyoxylate pathways following tributyrin supplementation. As far as we are aware, this is the first investigation focused on the effects of tributyrin supplementation on the gut microbiota of weaned piglets and demonstrating a relevant impact of this treatment on the composition and metabolic potential of fecal microbial communities. Thus, the beneficial effects of tributyrin may not be limited to supplementation of an extra source of energy to the enterocytes, which positively affects the integrity of the intestinal mucosa. Indeed, our results indicate that gut microbiota, in association with host-derived/related factors, may also play a role in exerting a positive impact on animal performances. The major limitation of this study is that the tested animals did not exhibit diarrhea episodes or health problems, yet our intent was to work in breeding conditions, without any challenge with pathogenic microorganisms. Nevertheless, the findings of the present study warrant further investigation to also elucidate the effects of tributyrin in artificial infection experiments mimicking swine enteric diseases.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/3/584/s1: Table S1: Diet compositions of *in vivo* trial (% as fed basis) divided per control (CTR) and treatment group fed with 0.2% of tributyrin (TRI). Table S2: Differentially abundant phyla and families between control and tributyrin groups of piglets. Table S3: Differentially abundant genera between tributyrin and control groups of piglets. Table S4: Summary table of r correlation value and corresponding p value of Spearman correlation analysis.

Author Contributions: Conceptualization, M.L.C., L.R., A.P. and L.M.; methodology, A.P., M.L.C. and V.P.; formal analysis, F.M., S.S., M.D. and A.S.; investigation, F.M., V.P., M.L.C. and S.S.; data curation, V.P., F.M., S.S., L.R., A.P. and M.L.C.; writing—original draft preparation, A.S., M.L.C., V.P. and F.M.; writing—review and editing, V.P., S.S., A.P. and L.R.; supervision, L.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the animal welfare body of University of Milan (authorization number 31/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequence files are available at the European Nucleotide Archive (ENA) database under the accession number PRJEB40653.

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References

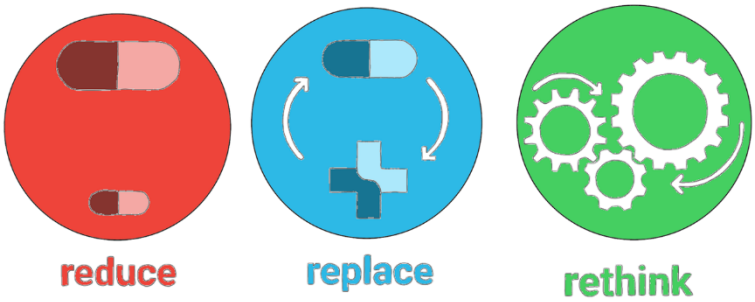
1. Guevarra, R.B.; Lee, J.H.; Lee, S.H.; Seok, M.J.; Kim, D.W.; Kang, B.N.; Johnson, T.J.; Isaacson, R.E.; Kim, H.B. Piglet gut microbial shifts early in life: Causes and effects. J. Anim. Sci. Biotechnol. **2019**, 10, 1–10.
2. Gao, J.; Yin, J.; Xu, K.; Li, T.; Yin, Y. What Is the Impact of Diet on Nutritional Diarrhea Associated with Gut Microbiota in Weaning Piglets: A System Review. Biomed Res. Int. 2019.
3. Mesonero-Escuredo, S.; Strutzberg-Minder, K.; Casanovas, C.; Segalés, J. Viral and bacterial investigations on the aetiology of recurrent pig neonatal diarrhoea cases in Spain. Porc. Health Manag. **2018**, 4, 1–6.
4. Ruiz, V.L.A.; Bersano, J.G.; Carvalho, A.F.; Catroxo, M.H.B.; Chiebao, D.P.; Gregori, F.; Miyashiro, S.; Nassar, A.F.C.; Oliveira, T.M.F.S.; Ogata, R.A.; et al. Case-control study of pathogens involved in piglet diarrhea Veterinary Research. BMC Res. Notes **2016**, 9.

5. Katsuda, K.; Kohmoto, M.; Kawashima, K.; Tsunemitsu, H. Frequency of enteropathogen detection in suckling and weaned pigs with diarrhea in Japan. J. Vet. Diagn. Investig. **2006**, 18, 350–354.
6. Dou, S.; Gadonna-Widehem, P.; Rome, V.; Hamoudi, D.; Rhazi, L.; Lakhal, L.; Larcher, T.; Bahi-Jaber, N.; Pinon-Quintana, A.; Guyonvarch, A.; et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS ONE **2017**, 12.
7. Yang, H.; Huang, X.; Fang, S.; He, M.; Zhao, Y.; Wu, Z.; Yang, M.; Zhang, Z.; Chen, C.; Huang, L. Unraveling the Fecal Microbiota and Metagenomic Functional Capacity Associated with Feed Efficiency in Pigs. Front. Microbiol. **2017**, 8, 1555.
8. Sun, J.; Du, L.; Li, X.L.; Zhong, H.; Ding, Y.; Liu, Z.; Ge, L. Identification of the core bacteria in rectums of diarrheic and non-diarrheic piglets. Sci. Rep. **2019**, 9, 1–10.
9. Boyen, F.; Haesebrouck, F.; Vanparys, A.; Volf, J.; Mahu, M.; Van Immerseel, F.; Rychlik, I.; Dewulf, J.; Ducatelle, R.; Pasmans, F. Coated fatty acids alter virulence properties of Typhimurium and decrease intestinal colonization of pigs. Vet. Microbiol. **2008**.
10. de Lange, C.F.M.; Pluske, J.; Gong, J.; Nyachoti, C.M. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livest. Sci. **2010**, 134, 124–134.
11. Thacker, P.A. Alternatives to antibiotics as growth promoters for use in swine production: A review. J. Anim. Sci. Biotechnol. **2013**, 4, 35.
12. EU Directive 1831/2003 of the European Parliament and of The Council on additives for use in animal nutrition. Off. J. Eur. Union **2003**, 268, 29–43.
13. 13. Hou, Y.Q.; Liu, Y.L.; Hu, J.; Shen, W.H. Effects of lactitol and tributyrin on growth performance, small intestinal morphology and enzyme activity in weaned pigs. Asian-Australas. J. Anim. Sci. **2006**, 19, 1470–1477.
14. Sotira, S.; Dell'Anno, M.; Caprarulo, V.; Hejna, M.; Pirrone, F.; Callegari, M.L.; Tucci, T.V.; Rossi, L. Effects of tributyrin supplementation on growth performance, insulin, blood metabolites and gut microbiota in weaned piglets. Animals **2020**, 10, 726.
15. Zhang, W.X.; Zhang, Y.; Zhang, X.W.; Deng, Z.X.; Liu, J.X.; He, M.L.; Wang, H.F. Effects of dietary supplementation with combination of tributyrin and essential oil on gut health and microbiota of weaned piglets. Animals **2020**, 10, 180.
16. Sakdee, J.; Poeikhampha, T.; Rakangthong, C.; Poungpong, K.; Bunchasak, C. Effect of tributyrin supplementation in diet on production performance and gastrointestinal tract of healthy nursery pigs. Pakistan J. Nutr. **2016**, 15, 954–962.
17. Wang, C.; Cao, S.; Shen, Z.; Hong, Q.; Feng, J.; Peng, Y.; Hu, C. Effects of dietary tributyrin on intestinal mucosa development, mitochondrial function and AMPK-mTOR pathway in weaned pigs. J. Anim. Sci. Biotechnol. **2019**, 10, 93.
18. Tugnoli, B.; Giovagnoni, G.; Piva, A.; Grilli, E. From acidifiers to intestinal health enhancers: How organic acids can improve growth efficiency of pigs. Animals **2020**, 10, 134.
19. Gu, Y.; Song, Y.; Yin, H.; Lin, S.; Zhang, X.; Che, L.; Lin, Y.; Xu, S.; Feng, B.; Wu, D.; et al. Dietary supplementation with tributyrin prevented weaned pigs from growth retardation and lethal infection via modulation of inflammatory cytokines production, ileal FGF19 expression, and intestinal acetate fermentation. J. Anim. Sci. **2017**, 95, 226–238.
20. Cao, S.T.; Wang, C.C.; Wu, H.; Zhang, Q.H.; Jiao, L.F.; Hu, C.H. Weaning disrupts intestinal antioxidant status, impairs intestinal barrier and mitochondrial function, and triggers mitophagy in piglets. J. Anim. Sci. **2018**, 96, 1073–1083.

21. Kovanda, L.; Zhang, W.; Wei, X.; Luo, J.; Wu, X.; Atwill, E.R.; Vaessen, S.; Li, X.; Liu, Y. *In vitro* antimicrobial activities of organic acids and their derivatives on several species of Gram-negative and Gram-positive bacteria. Molecules **2019**, 24, 3770.
22. Namkung, H.; Yu, H.; Gong, J.; Leeson, S. Antimicrobial activity of butyrate glycerides toward Salmonella Typhimurium and Clostridium perfringens. Poult. Sci. **2011**, 90, 2217–2222.
23. McCormack, U.M.; Curião, T.; Buzoianu, S.G.; Prieto, M.L.; Ryan, T.; Varley, P.; Crispie, F.; Magowan, E.; Metzler-Zebeli, B.U.; Berry, D.; et al. Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. Appl. Environ. Microbiol. **2017**, 83.
24. McCormack, U.M.; Curião, T.; Metzler-Zebeli, B.U.; Magowan, E.; Berry, D.P.; Reyer, H.; Prieto, M.L.; Buzoianu, S.G.; Harrison, M.; Rebeiz, N.; et al. Porcine Feed Efficiency-Associated Intestinal Microbiota and Physiological Traits: Finding Consistent Cross-Locational Biomarkers for Residual Feed Intake. mSystems **2019**, 4.
25. NCR. Nutrient Requirements of Swine, 11th ed.; The National Academies Press: Washington, DC, USA, 2012.
26. Patrone, V.; Minuti, A.; Lizier, M.; Miragoli, F.; Lucchini, F.; Trevisi, E.; Rossi, F.; Callegari, M.L. Differential effects of coconut versus soy oil on gut microbiota composition and predicted metabolic function in adult mice. BMC Genom. **2018**, 19.
27. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics **2014**, 30, 2114–2120.
28. Li, H.; Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics **2010**, 26, 589–595.
29. Chong, J.; Liu, P.; Zhou, G.; Xia, J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat. Protoc. **2020**, 15, 799–821.
30. Dhariwal, A.; Chong, J.; Habib, S.; King, I.L.; Agellon, L.B.; Xia, J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. **2017**, 45, W180–W188.
31. Clavel, T.; Desmarchelier, C.; Haller, D.; Gérard, P.; Rohn, S.; Lepage, P.; Daniel, H. Intestinal microbiota in metabolic diseases: From bacterial community structure and functions to species of pathophysiological relevance. Gut Microbes **2014**, 5, 544–551.
32. Looft, T.; Bayles, D.O.; Alt, D.P.; Stanton, T.B. Complete genome sequence of Coriobacteriaceae strain 68-1-3, a novel mucusdegrading isolate from the swine intestinal tract. Genome Announc. **2015**, 3.
33. Tailford, L.E.; Crost, E.H.; Kavanaugh, D.; Juge, N. Mucin glycan foraging in the human gut microbiome. Front. Genet. **2015**, 5, 81.
34. Chen, J.; Wright, K.; Davis, J.M.; Jeraldo, P.; Marietta, E.V.; Murray, J.; Nelson, H.; Matteson, E.L.; Taneja, V. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome Med. **2016**, 8, 43.
35. Leclercq, S.; Matamoros, S.; Cani, P.D.; Neyrinck, A.M.; Jamar, F.; Stärkel, P.;Windey, K.; Tremaroli, V.; Bäckhed, F.; Verbeke, K.; et al. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. Proc. Natl. Acad. Sci. USA **2014**, 111, E4485–E4493.
36. Mokkala, K.; Röytiö, H.; Munukka, E.; Pietilä, S.; Ekblad, U.; Rönnemaa, T.; Eerola, E.; Laiho, A.; Laitinen, K. Gut Microbiota Richness and Composition and Dietary Intake of Overweight Pregnant Women Are Related to Serum Zonulin Concentration, a Marker for Intestinal Permeability. J. Nutr. **2016**, 146, 1694–1700.

37. Chen, W.; Liu, F.; Ling, Z.; Tong, X.; Xiang, C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PLoS ONE **2012**, 7.
38. Berding, K.; Wang, M.; Monaco, M.H.; Alexander, L.S.; Mudd, A.T.; Chichlowski, M.;Waworuntu, R.V.; Berg, B.M.; Miller, M.J.; Dilger, R.N.; et al. Prebiotics and bioactive milk fractions affect gut development, microbiota, and neurotransmitter expression in piglets. J. Pediatr. Gastroenterol. Nutr. **2016**, 63, 688–697.
39. Burrough, E.R.; Arruda, B.L.; Plummer, P.J. Comparison of the luminal and mucosa-associated microbiota in the colon of pigs with and without swine dysentery. Front. Vet. Sci. **2017**, 4.
40. Nakazawa, F.; Poco, S.E.; Sato, M.; Ikeda, T.; Kalfas, S.; Sundqvist, G.; Hoshino, E. Taxonomic characterization of Mogibacterium diversum sp. nov. and Mogibacterium neglectum sp. nov., isolated from human oral cavities. Int. J. Syst. Evol. Microbiol. **2002**, 52, 115–122.
41. Liu, J.H.; Zhang, M.L.; Zhang, R.Y.; Zhu,W.Y.; Mao, S.Y. Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. Microb. Biotechnol. **2016**, 9, 257–268.
42. Tan, Z.; Wang, Y.; Yang, T.; Ao, H.; Chen, S.; Xing, K.; Zhang, F.; Zhao, X.; Liu, J.; Wang, C. Differences in gut microbiota composition in finishing Landrace pigs with low and high feed conversion ratios. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **2018**, 111, 1673–1685.
43. Ding, X.; Lan, W.; Liu, G.; Ni, H.; Gu, J.D. Exploring possible associations of the intestine bacterial microbiome with the pre-weaned weight gaining performance of piglets in intensive pig production. Sci. Rep. **2019**, 9, 1–10.
44. Kubasova, T.; Davidova-Gerzova, L.; Babak, V.; Cejkova, D.; Montagne, L.; Le-Floc'h, N.; Rychlik, I. Effects of host genetics and environmental conditions on fecal microbiota composition of pigs. PLoS ONE **2018**, 13, e0201901.
45. Gibson, G.R.; MacFarlane, G.T.; Cummings, J.H. Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut **1993**, 34, 437–439.
46. Gardiner, G.E.; Metzler-Zebeli, B.U.; Lawlor, P.G. Impact of intestinal microbiota on growth and feed efficiency in pigs: A review. Microorganisms **2020**, 8, 1886.
47. Lam, Y.Y.; Ha, C.W.Y.; Campbell, C.R.; Mitchell, A.J.; Dinudom, A.; Oscarsson, J.; Cook, D.I.; Hunt, N.H.; Caterson, I.D.; Holmes, A.J.; et al. Increased Gut Permeability and Microbiota Change Associate with Mesenteric Fat Inflammation and Metabolic Dysfunction in Diet-Induced Obese Mice. PLoS ONE **2012**, 7, e34233.
48. Million, M.; Tomas, J.; Wagner, C.; Lelouard, H.; Raoult, D.; Gorvel, J.P. New insights in gut microbiota and mucosal immunity of the small intestine. Hum. Microbiome J. **2018**, 7–8, 23–32.
49. Belzer, C.; Gerber, G.K.; Roeselers, G.; Delaney, M.; DuBois, A.; Liu, Q.; Belavusava, V.; Yeliseyev, V.; Houseman, A.; Onderdonk, A.; et al. Dynamics of the microbiota in response to host infection. PLoS ONE **2014**, 9.
50. Chen, Y.R.; Zheng, H.M.; Zhang, G.X.; Chen, F.L.; Chen, L.D.; Yang, Z.C. High Oscillospira abundance indicates constipation and low BMI in the Guangdong Gut Microbiome Project. Sci. Rep. **2020**, 10, 1–8.
51. Tigchelaar, E.F.; Bonder, M.J.; Jankipersadsing, S.A.; Fu, J.; Wijmenga, C.; Zhernakova, A. Gut microbiota composition associated with stool consistency. Gut **2016**, 65, 540–542.
52. Gophna, U.; Konikoff, T.; Nielsen, H.B. Oscillospira and related bacteria—From metagenomic species to metabolic features.

6 Alternative model to animal experimentation for studying nutrient bioaccessibility



In the frame of this project, an alternative model for the assessment of nutrient bioaccessibility was developed. The aim was to collect reliable data before *in vivo* testing of ingredients, additives and functional compounds for reducing and replacing animals for experimental purpose. Considering this, an oral presentation was given at the 72nd Annual Meeting of the European Federation of Animal Science held in Davos (Switzerland).

6.1 *Ex vivo* intestinal model for the evaluation of nutrient bioaccessibility

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Nowadays, the nutrition plays a pivotal role for both human and animal health, and increasingly requires science-based evidences to develop effective functional diets. In order to reduce the use of animals for experimental purposes innovative reliable and informative models, simulating the complex intestinal physiology, represent an expanding research field. In this scenario, the aim of this study was to develop and evaluate an intestinal swine *ex vivo* model for the evaluation of nutrient bioaccessibility and its functionality among time. A pig intestine was harvested, following Maastricht criteria, from a 100 kg pig at the slaughterhouse. The isolated duodenum tract was reperfused in normothermic conditions with heterologous blood after cold ischemic storage. *Ex vivo* duodenum perfusion model was maintained in controlled pressure conditions (flux pump 1.48 L/min, O_2 2 L/min, artery pressure 76/55 mmHg) through extracorporeal circulation for 4 hours. During this period, blood and luminal content samples were collected at regular intervals for the evaluation of the concentrations of glucose by glucometer, minerals (Na, Ca, Mg, K, P) by ICP-AES, lactate-dehydrogenase (LDH) and derivates of reactive oxygen metabolites (D-ROMs) by colorimetric methods. Histological examination was carried on three intestinal

segments. The macroscopic observation showed peristaltic activity caused by intrinsic nerves. Measured glucose levels suggested its utilization at cellular level confirming the organ viability in line with histological examination. At the end of the experimental period, intestinal mineral concentrations resulted lower than their level in blood plasma highlighting their bioaccessibility ($p < 0.001$). A progressive increase of D-ROMs and LDH concentration among time, in luminal content, was observed related to a slight oxidative stress ($p < 0.05$). Isolated *ex vivo* duodenum perfusion developed model fulfils all the prerequisites to study bioaccessibility of nutrients, offering a variety of experimental possibilities in line with 3Rs principles.

Brief Introduction to the Study:

The following manuscript is one of the most interesting innovations regarding the *ex vivo* evaluation of nutrient bioaccessibility. The model proposes a pioneering perfused duodenum tract model for nutritional studies of nutrients kinetics and absorption. The importance of the study relies on the fact that no animals for experimental purposes are needed in the following model, and the high potential for providing strong science-based data prior to *in vivo* testing without the needing of animals. Limitations rely on the absence of hormonal regulation, the lack of a gold standard and the complex surgery technique that requires an important ability and expertise. To date, the model is still expensive and a large scale-up is required in order to provide enough replicates for supporting robust statistical findings. However, the model potentiality is enormous in line with 3R principles on animal experimentation and for the translational applications to human field regarding organ transplantation.

6.2 Swine intestinal segment perfusion model for the evaluation of nutrients bioaccessibility

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Abstract

The nutrition science requires more science-based evidences for the development of effective functional diets. To reduce animals for experimental purposes innovative reliable and informative models, simulating the complex intestinal physiology, are needed. The aim of this study was to develop a swine duodenum segment perfusion model for the evaluation of nutrient bioaccessibility and functionality among time. At slaughterhouse, one sow intestine was harvested from 100 kg following Maastricht criteria for cardiac death organ donation (CDC) for transplantation purposes. Duodenum tract was isolated and perfused in sub-normothermic conditions with heterologous blood after cold ischemia induction. Duodenum segment perfusion model was maintained under controlled pressure conditions through extracorporeal circulation for 3 hours. Blood samples from extracorporeal circulation and luminal content samples were collected at regular intervals for the evaluation of glucose

concentration by glucometer, minerals (Na^+, Ca^{2+}, Mg^{2+}, K^+) by ICP-OES, lactate-dehydrogenase and nitrite oxide by spectrophotometric methods. The macroscopic observation showed peristaltic activity caused by intrinsic nerves. Glycemia decreased over time (from 44.00±1.20 mg/dL to 27.50±0.41; $p < 0.01$), suggesting glucose utilization by the tissue confirming the organ viability in line with histological examinations. At the end of the experimental period, intestinal mineral concentrations resulted lower than their level in blood plasma suggesting their bioaccessibility ($p < 0.001$). A progressive increase of LDH concentration over time was observed in the luminal content probably related to a loss of viability (from 0.32±0.02 to 1.36±0.02 OD; $p < 0.05$) confirmed by histological findings that revealed a de-epithelization of the distal portion of duodenum. Isolated swine duodenum perfusion model satisfied the criteria for studying bioaccessibility of nutrients, offering a variety of experimental possibilities in line with 3Rs principle.

Keywords: duodenum, intestinal segment, nutrient bioaccessibility, absorption, swine, alternative models, swine gut, absorption.

1. Introduction

Nowadays, the nutrition plays a pivotal role for both human and animal health, related not only to simply to satisfy nutritional requirements, it also plays a key role in the health and welfare also through its functionality (Ross et al., 2012; Domínguez Díaz et al., 2020; Novik and Savich, 2020; Hull, 2021). The literature offers an heterogenous panorama of nutritional studies, and several studies are contradictory on the argument. The prediction of the nutritional quality of food and feed products requires more knowledge related to the individual digestibility of dietary compounds (Devle et al., 2014).

Currently, the request to reduce animals for experimental purposes is continuously growing. European policies on animal experimentation are intensely aiming to increase the protection of experimental animals, thus various alternative methods have being developed to achieve this purpose (Jin et al., 2020). Even the most advanced *in vitro* and *in silico* systems cannot fully simulate complex phenomena such as inflammation, digestion, pathologies, and metabolism. However, the introduction of innovative non-animal models is fundamental as complementary to animal experimentation (Griffiths et al., 2020). Thus, reliable science-based models are required in order to reduce and replace animals for experimental purposes. The gastrointestinal physiology is a complex field that involves different tissues and

systems (epithelium, muscles, nerves, connective, hormones and glandule), and the use of animals for studying nutrient digestion is still considered essential.

Though remarkable results achieved with *in vitro* models, the organ architecture, the epithelium integrity and the nutrient absorption are not, at present, efficiently simulated. 3D organoids developed for simulating the gastrointestinal physiology cannot mimic the complete environment due to the fact that microbiota belongs to the host as a natural flora. The *ex vivo* digestion models are supposed to mimic the *in vivo* processes better than the *in vitro* technology, thanks to a manufacturing process of digestive fluids, and the presence of the complete array of proteases and substances that *in vivo* concur to the digestive process (Lueangsakulthai et al., 2020). Several *ex vivo* studies have been conducted on pigs due to the similar characteristics of swine species in terms of similar morphology, structure, composition and enzymatic activity to humans and the low cost of this technology.

Organ explant efficiently simulate the entire animal complexity, offering the possibility to perform *ex vivo* experiments under standardized conditions and harvest several tissues from a single animal in line with the 3R principle (replace, reduce, refine). In addition, the possibility to perform more tests involving the same donor, improves the robustness of the *ex vivo* models (Baydoun et al., 2020). It has been demonstrated that the mucosal epithelium is extremely susceptible to ischemia (Salehi et al., 2003). The perfusion of *ex vivo* tissue allow to the conservation of organ viability and structure for a longer period compared to classic *ex vivo* models (Stangl et al., 2000). Recent studies, showed encouraging results of swine intestinal perfused model suggesting it as a cost-effective, practical and reliable strategy for the study of intestinal physiology, pharmacology and transplantation (Hamed et al., 2021). In this scenario, the aim of this study was to develop and assess a swine intestinal segment perfusion model for the evaluation of nutrient bioaccessibility and organ functionality among time for further applications to study the effect of feed ingredients and additives on nutrients bioavailability.

2. Materials and Methods

2.1 Organ harvesting and extracorporeal circulation

One gastrointestinal tract was harvested at the slaughterhouse from a 100kg Large White sow, following the Maastricht criteria for cardiac death organ donation (CDC) for transplantation purposes (Thuong et al., 2016). Heterologous blood was collected at the slaughterhouse and 25 IU of heparin and 1 g of Cefazolin were added to avoid coagulation and bacterial contaminations. The blood was stored at 4 °C until organ perfusion. During the period of the "warm ischemia", that lasted from the animal

exsanguination until the duodenum isolation, the mesenteric artery was cannulated (Lauronen et al., 2001). The "cold ischemia" started with arterial infusion of 2 L of Belzer UW Cold Storage solution (S.A.L.F. Spa, Bergamo, Italy) (Guibert et al., 2011) within 9 minutes for organ structure preservation. The descendant part of duodenum was isolated, and an intraluminal access was set-up by fixing a luer-lock connector on the pylorus side. The intestinal segment was intraluminal perfused with 360 mL of sterile Krebs-Ringer buffer (NaCl 115 mmol/L, K_2HPO_4 2.4 mmol/L, KH_2PO_4 0.4 mmol/L, $CaCl_2$ 1.2 mmol/L, $MgCl_2$ 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, glucose 10 mmol/L) according to Biolley et al. (2019) for favoriting the epithelial structure conservation. The organ was transported to the laboratory submerged in a Ringer's lactate solution at 4°C. Duodenum tract was maintained in controlled pressure conditions (flux pump 1.48 L/min, O_2 2 L/min, artery pressure 76/55 mmHg) through extracorporeal circulation with heterologous blood at sub-normothermic perfusion condition for 3 hours starting by the addition of warm blood at 37 °C. The circuit was composed by a peristaltic pump (as a beating heart), venous reservoir and blood oxygenator (as lungs) (Alexandre and Fabiani, 2010). Organ temperature was monitored using a thermometer for the entire experimental period.

2.2. Sample collection and analyses

During the first hour of extracorporeal circulation blood samples were collected from the line at 7 minutes interval (T0, T1, T2, T3, T4, T5, T6, T7, T8). Throughout the second hour, hematic samples were collected each 15 minutes (T9, T10, T11, T12) and at the third hour the collection was performed at 30 minutes intervals (T13, T14). Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4 °C. In addition, the intestinal content was collected from T0 to T14 every 30 minutes from the luer-lock connector. All biological samples were evaluated for mineral and metabolites concentrations.

Glucose concentration was evaluated immediately after blood and intestinal lumen solution sampling from extracorporeal circulation line and the luer-lock connector using a glucometer (U-Right 4278, Biochemical Systems International S.p.A, Arezzo, Italy). The content of main macro-elements (Na^+, K^+, Mg^{2+}, Ca^{2+}) was assessed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Optima 3300 XL, Perkin Elmer Inc., USA). First, calibration curves for each element considered were obtained using certified reference materials. Blood and intestinal content samples were then diluted 1:100 (v/v) with sterile MilliQ water, filtered with 0.45 um syringe filters and injected. The pH of intestinal lumen content was evaluated with pHmeter. Lactate dehydrogenase (LDH) and nitrite ion (NO_2^-) concentrations were evaluated through colorimetric kits (CytoTox 96® Non-Radioactive Cytotoxicity Assay and Griess Reagent System, Promega Italia S.r.l, Milan, Italy) following the manufacturer

instructions. In particular, absorbances were red at 490 nm for LDH and 540 nm for NO_2^- using a spectrophotometer (Model 680 Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Histological evaluation

After 3 hours of continuous perfusion, the intestinal segment was subdivided in three portions (proximal A, medial B, and distal C; Figure 1) and tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E).

To assess the extent of epithelial cells proliferation in the intestinal mucosa, immunohistochemistry with anti-ki67 (SP6, RM-9106-S, Labvision) primary antibody was performed. Four μm sections were deparaffinized and underwent heat-induced epitope retrieval at pH 9, for 40 min at 95°C (Dewax and HIER Buffer H, TA-999-DHBH, Thermo Scientific, UK). Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 10 min. Slides were rinsed and treated with PBS containing 10% normal serum for 30 min to reduce nonspecific background staining and then incubated for 1 hour at room temperature with the primary antibodies. Sections were incubated for 30 min with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA), and then labelled by the avidin-biotin-peroxidase (ABC) procedure with a commercial immunoperoxidase kit (VECTASTAIN Elite ABC HRP Kit Standard, PK-6100, Vector Laboratories, Burlingame, CA, USA). The immunoreaction was visualized with 3,3'-diaminobenzidine substrates (Peroxidase DAB Substrate Kit, VC-SK-4100-KI01, Vector Laboratories, Burlingame, CA, USA) for 5 min and sections were counterstained with Mayer's hematoxylin. Digital slides were obtained from H&E, and immunostained sections by using the NanoZoomer-S60 Digital slide scanner (Hamamatsu, C13210-01), and images were captured by using theNDP.view2 Viewing software (Hamamatsu, U12388-01).

To assess the proliferation of the intestinal mucosa, the % of ki67-positive epithelial cells (number of ki67 positive nuclei/number of total nuclei x 100) was evaluated using the ImageJ analysis program (http://rsb.info.nih.gov/ij/).

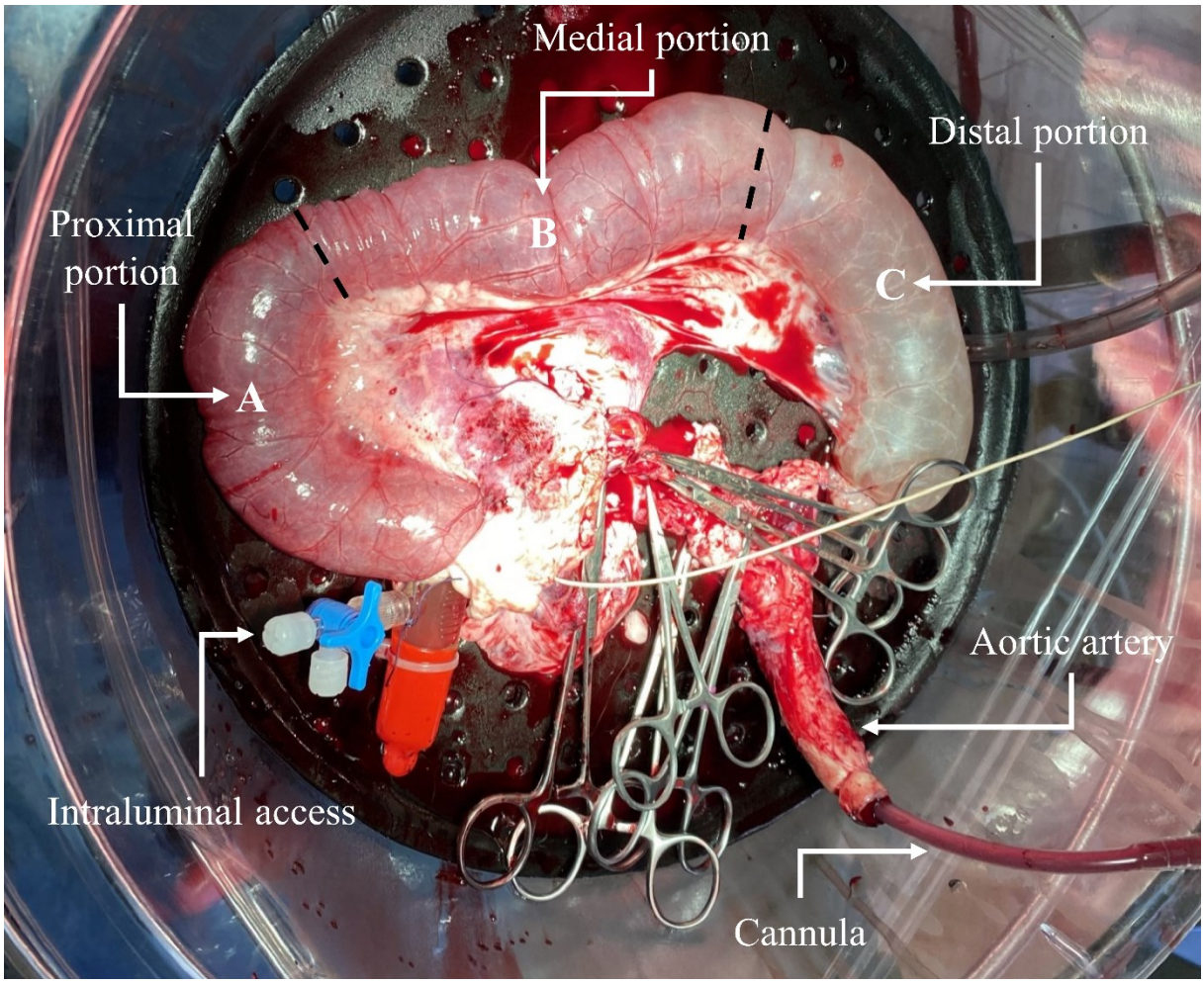

Figure 1. Representative image of the intestinal segment perfusion model, ideally subdivided in proximal (A), medial (B) and distal (C) portions for tissue sampling. The outlined lines indicate the incision points.

2.4. Statistical analysis

The results of blood and intestinal content glucose, minerals and metabolites were analyzed using a one-way ANOVA using JMP® Pro 15 (SAS Inst. Inc., Cary, NC, USA). Multiple comparisons among timepoints were evaluated by performing Tukey's Honestly Significant Difference test (Tukey's HSD). The results were presented as least square means ± standard errors (SE). The means were considered different when $p \leq 0.05$.

3. Results

During the extracorporeal circulation, pressure and oxygenation conditions were maintained stable showing no alterations for three hours. Organ temperature was progressively increasing over time, maintaining the sub-normothermic perfusion throughout the experimental period (T0 = 15.2 °C, T1 = 17.5 °C, T2 = 18.8 °C, T3 = 19.2

°C, T4 = 20.5 °C, T5 = 21.0 °C, T6 = 22.0 °C, T7 = 23.0 °C, T8 = 23.6 °C, T9 = 24.5 °C, T10 = 25.0 °C, T11 = 25.0 °C, T12 = 25.6 °C, T13 = 25.6 °C, T14 = 25.8 °C). In general, the intestinal segment showed redness due to blood vessels reperfusion, except in the distal portion C that appeared pale and bloodless, and the peristaltic activity was observed after pinching stimulation. Reaching 25.3 °C the peristaltic contraction was noted without any external stimulation.

3.1 Glucose, mineral concentrations, organ temperature and intestinal pH

The glucose level measured in Krebs-Ringer solution was 123.67±2.31 mg/dL. The glycemia revealed a constant decreasing trend from an initial concentration of 44.00±1.20 to 27.50±0.41 mg/dL after 3 hours of extracorporeal circulation (Figure 2). Considering the sampling intervals, during the first hour (T0-T8) statistically significant different concentrations were registered comparing T0 to T5, T6, T7 and T8 (44.00±1.20, 36.50±1.20, 41.50±1.20, 38.25±1.20, 38.50±1.20 mg/dL respectively ($p < 0.001$). In the course of the second (T8-T12) and third hour (T12-T14) glucose concentration gradually decreased until the end of experiment ($p < 0.01$). The glucose concentrations of intestinal lumen did not significantly differ from the beginning to the end of extracorporeal circulation (T0: 94.00±6.43 mg/dL; T4: 99.50±6.43 mg/dL; T8: 97.50±6.43; T12: 90.50±4.55 mg/dL; T14: 84.00±5.25).

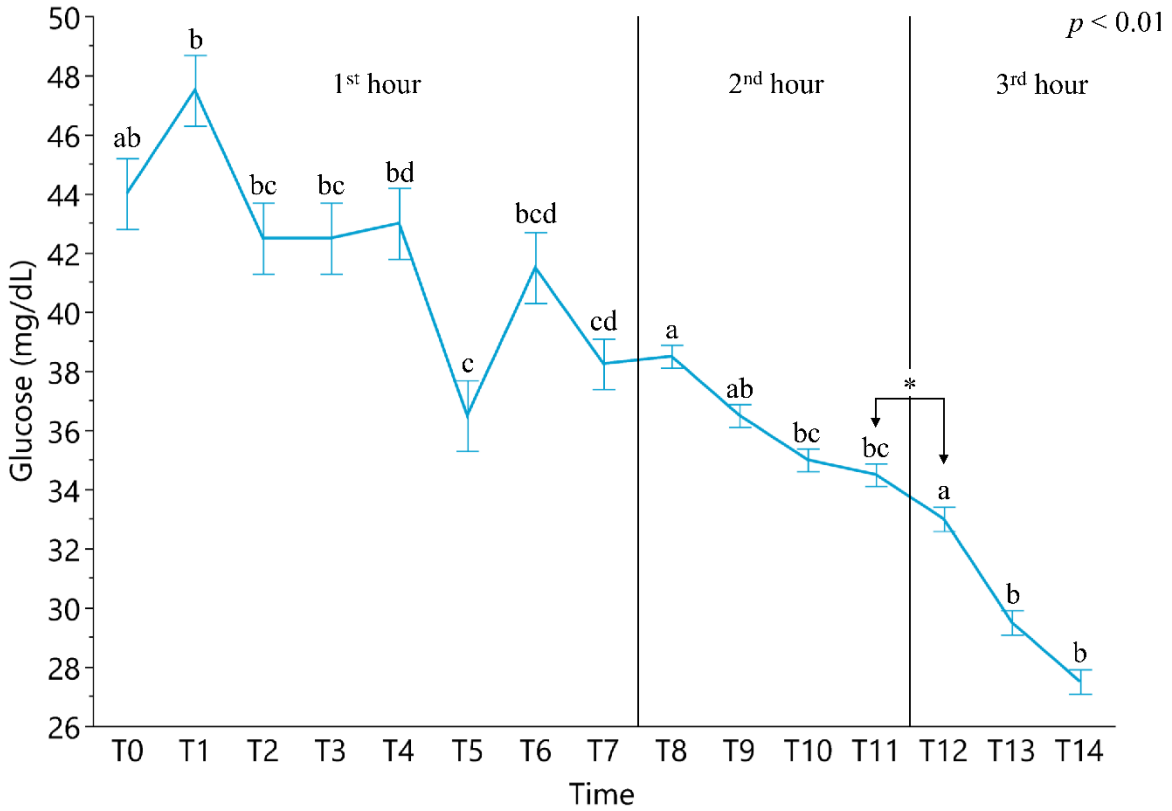

Figure 2. Glycaemic levels measured for 3 hours from the extracorporeal circulation of perfused intestinal segment.

Data are expressed as least squared means and standard errors.
Different lowercase letters indicate statistically significant differences ($p \leq 0.01$).
First hour: hematic glucose concentrations of the first hour measured at 7 minutes intervals; Second hour: hematic glucose concentrations of the second hour measured at 30 minutes intervals; Third hour: hematic glucose concentrations of the third hour measured at 30 minutes intervals. Asterisk indicates statistically significant different values.

Considering the mineral concentrations, Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} showed significant differences over the experimental time (Figure 3). In particular, Na^{+} blood levels raised from T0 to T4 and decreased slightly at T14, while its concentration in intestinal content was constantly reducing over time ($p < 0.01$). Ca^{2+}, Mg^{2+}, K^{+} blood concentrations increased significantly over time, and co-currently their concentrations in the intestinal lumen significantly dropped from T0 to T14 ($p < 0.01$).
Intestinal pH showed a constant value of 7 registered for T0, T4, T8, T12 and T14.

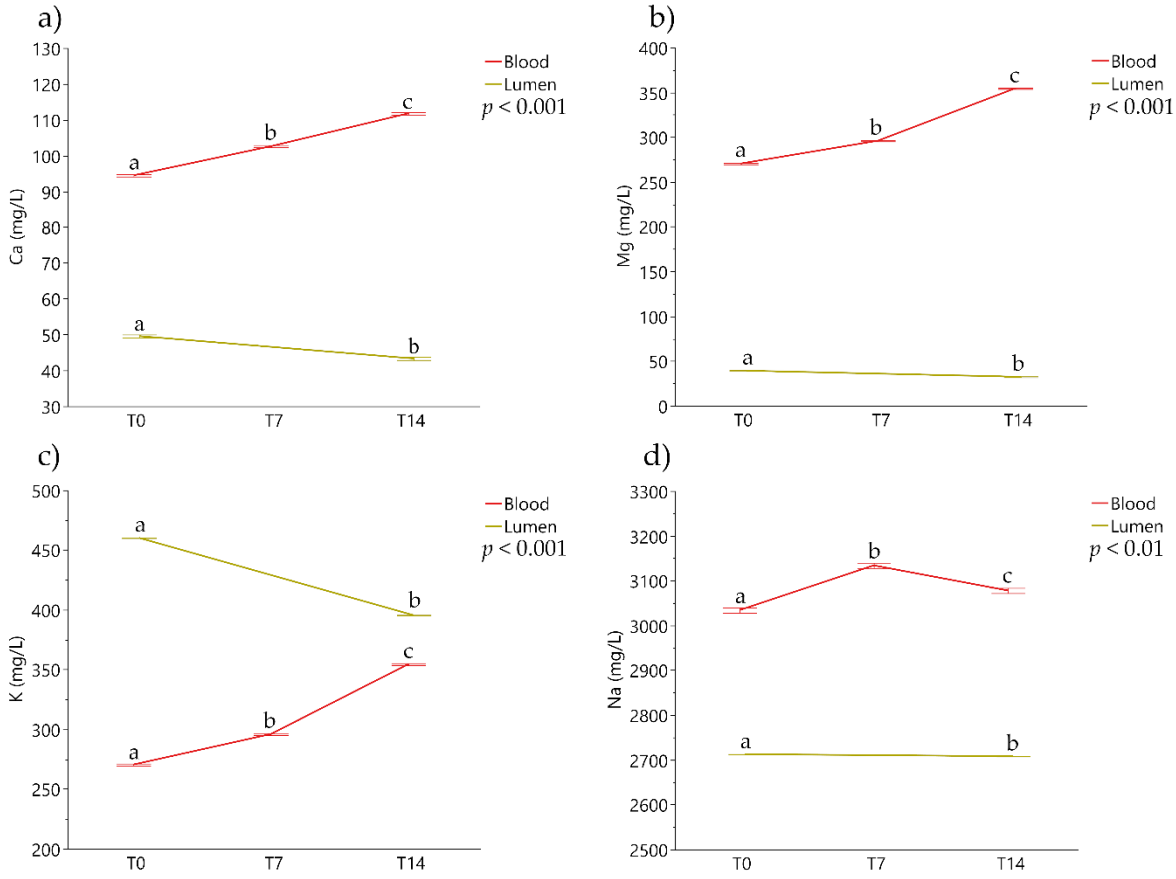

Figure 3. Mineral concentrations measured over time in blood plasma and intestinal lumen content.
Data are expressed as least squared means and standard errors.
Different lowercase letters indicate statistically significant differences ($p \leq 0.05$).
a) Calcium (Ca^{2+}) concentrations measured at T0-T7-T14 in blood and T0-T14 in intestinal lumen content; b) Magnesium (Mg^{2+}) concentrations measured at T0-T7-T14 in blood and T0-T14 in intestinal lumen content; c) Potassium (K^{+}) concentrations measured at T0-T7-T14 in blood and T0-T14 in

intestinal lumen content; d) Sodium (Na^+) concentrations measured at T0-T7-T14 in blood and T0-T14 in intestinal lumen content.

3.2. Lactate dehydrogenase and nitrite concentrations

LDH blood concentrations revealed a persistent trend over time even if statistically significant differences were observed in T3 and T4 compared to T14 (T3: 0.77±0.05 OD, T4: 0.85±0.05 OD and T4: 1.08±0.05 OD; $p < 0.05$; Table 1). Intestinal lumen content registered raising concentrations of LDH from T4 to T14 ($p < 0.0001$).

Nitrite ion concentrations were below the detection level of the assay for the entire experimental period (< 1.56 μM).

Table 1. Lactate dehydrogenase concentrations measured over time in blood and intestinal lumen content.

Timepoint	Blood (OD)	Lumen (OD)
T0	0.93±0.05^{ab}	0.32±0.02^{a}
T1	0.99±0.05^{ab}	
T2	0.81±0.05^{ab}	
T3	0.77±0.05^{a}	
T4	0.84±0.05^{a}	0.34±0.02^{a}
T5	0.87±0.05^{ab}	
T6	0.82±0.05^{ab}	
T7	0.89±0.05^{ab}	
T8	0.89±0.05^{ab}	0.50±0.02^{b}
T9	0.86±0.05^{ab}	
T10	0.92±0.05^{ab}	
T11	0.83±0.05^{ab}	
T12	0.92±0.05^{ab}	0.80±0.02^{c}
T13	1.01±0.05^{ab}	
T14	1.08±0.05^{b}	1.36±0.02^{d}
p-value	0.0256	< 0.0001

Data are expressed as least squared means and standard errors.
Different lowercase letters indicate statistically significant differences ($p \leq 0.05$).
OD: optical densities measured at 490 nm of wavelength.

3.3. Histological evaluation of the intestinal segment

In all examined portions (A, B, C), the intestinal architecture was preserved. A diffuse moderate infiltration of lymphocytes, plasma cells and lesser numbers of neutrophils was present in the intestinal mucosa, consistent with a diffuse moderate subacute enteritis. Moderate hyperemia was present in portions A and B (Figure 4). The intestinal epithelium of A and B portions were similar in terms of a slight de-epithelialization in the apical part of villi (Figure 4A, Figure 4B). On the contrary, the intestinal portion C showed absence of erythrocytes within blood vessels (impaired perfusion) and a diffuse necrosis of villi was visible (Figure 4C). Immunohistochemistry analysis revealed 47.58% of proliferating epithelial cells in portion A, 35.01% in portion B and 33.72% in portion C (Figure 5).

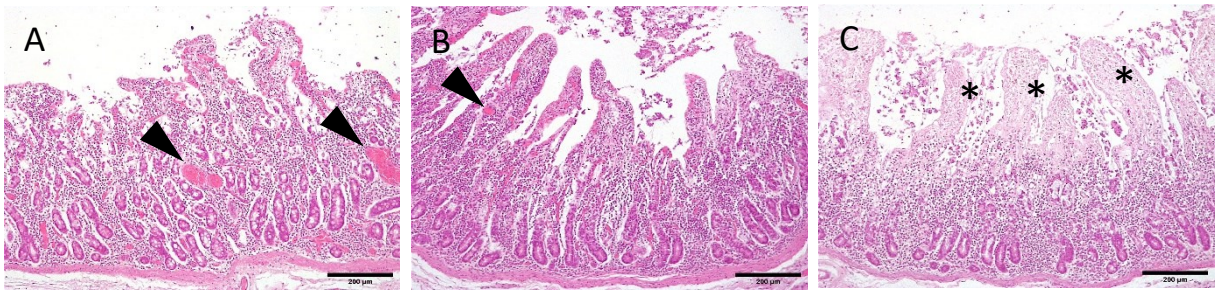

Figure 4. Histology of the perfused intestinal tract at the end of the 3 hours of extracorporeal circulation (H&E stain, 100x, scale bar = 200 μm). Portions A and B were overall well preserved and perfused, as demonstrated by blood vessels engorged with erythrocytes (arrowheads). In portion C, blood vessels are not evident (impaired perfusion) and necrosis of villi (*) was present. Haematoxylin eosin of intestinal A, B and C sections.

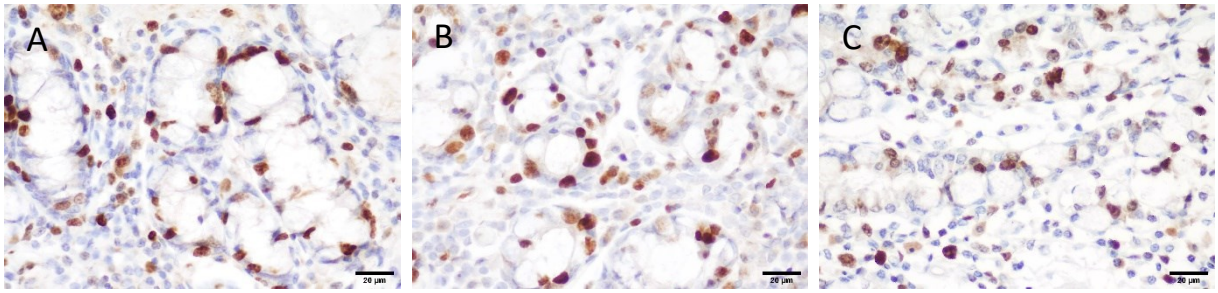

Figure 5. Proliferation of intestinal crypt epithelial cells. Immunoperoxidase staining for ki67, 400x, scale bar = 20 μm in portions A, B, and C.

4. Discussion

This study aimed to investigate the possibility to develop an alternative perfused intestinal segment model to study the nutrient bioaccessibility and bioavailability targeting to provide more complete data compared to other *in vitro* models. It is important to consider that this model is more advanced if compared to a classic *ex vivo*

model which do not provide extracorporeal circulation. This model was obtained following the surgery techniques used for *in vivo* organ transplantations in order to preserve the organ viability and architecture during the entire experimental period (Stangl et al., 2000).

Glucose bioaccessibility was evaluated due to its relevance *in vivo* as energetic marker derived from starch and sugar digestion. The glucose level is considered a marker of cell proliferation, viability (Li et al., 2007; MacIver et al., 2008) and functionality for the metabolic activity (Clara et al., 2017). The initial glycemic level (44.00±1.20 mg/dL) could be considered low if compared to the normal range of swine species (from 80 to 120 mg/dL) (Uc et al., 2015; Lombardi et al., 2020). The observed initial low glycaemia could be due to the animal fasting before slaughtering procedures. The glycemic trend showed a decreasing curve suggesting the utilization of glucose by erythrocytes and intestinal cells and the impossibility to store sugars in the intestinal tissue after blood perfusion. We observed a peak of glucose absorption at 7 minutes of extracorporeal circulation, and after the first hour a drop of 18% was registered. Even without registering significant differences, endoluminal content of glucose showed decreasing concentrations probably due to the low amount of glucose consumed by erythrocytes for their metabolism (Nossol et al., 2015). However, the observed results suggested that glucose was transported throughout its traditional mechanism. Glucose uptake is mediated via the Sodium-Glucose Cotransporter 1 (SGLT1) localized on the enterocytes membrane and its basolateral to the blood circulation is mediated primarily by the Glucose Transporter 2 (GLUT2) (Croset et al., 2001). Glucose absorption is a complex mechanism also mediated by pancreas and renin-angiotensin-aldosterone (Giacchetti et al., 2005). In this study, the glucose absorption could not be mediated by hormonal asset (Khan and Pessin, 2002) since it can be fully replicated only *in vivo.* The glucose concentration used was based on the defined level for the Krebs-Ringer solution as intraluminal nutrient medium. The low intraluminal glucose concentration (Verhoeckx et al., 2015) permitted to confirm organ metabolism without registering osmotic damage (Quan et al., 2008), even if it was not possible to evaluate a kinetic curve of its uptake (Hamed et al., 2021). Observed results suggest that the glucose could be considered an interesting marker for further application of this perfusion model for the evaluation of functional feed additives which are meant to improve gut health and nutrient utilization (Sotira et al., 2020; Miragoli et al., 2021).

Minerals are essential inorganic nutrients that have to be exclusively acquired from the diet (Samat et al., 2020). Minerals concentration was evaluated as indirect indicator of intestinal function as they do not require digestion to be absorbed. Na^+ concentration in blood displayed a significant increase from T0 to T7 and decreased from T7 to T14. In the meantime, the intestinal lumen content of Na^+ dropped

significantly from T0 to T14. The observed increase in osmolarity suggested that Na^+ uptake and utilization was maintained during the extracorporeal circulation. The absorption of Na^+ is associated with glucose uptake from Sodium-Glucose Transporters (SGLT1) (Navale and Paranjape, 2016). In this model, the physiologic separation of vascular, interstitial, and intracellular sections was conserved, and the blood concentration of Na^+ required to maintain a correct balance of its level in the interstitial matrix.

K^+ is one of the most important minerals for the acid-base and osmotic pressure balance (Baloš et al., 2016). K^+ is the most abundant cation in the intracellular and its plasma level is lower than sodium abundance (Udensi and Tchounwou, 2017). Similar to what observed for Na^+, K^+ levels increased in blood and decreased in lumen content from T0 to T14.

Mg^{2+} is one of widely abundant minerals in the animal body, and it is involved in several pivotal processes such as energy production, muscular contraction and nervous impulses transferring (Gröber et al., 2015). The observed increased Mg^{2+} plasma concentration suggests its absorption from intestinal lumen. In our study, Mg^{2+} blood level showed a drop from T0 to T7 probably due to epithelial utilization of this mineral. Subsequently, the Mg^{2+} concentration increased from T7 to T14 indicating its absorption from the lumen content. The intestinal lumen levels of Mg^{2+} significantly dropped from T0 to T14 indicating a duodenal uptake through passive and facilitated diffusion processes (Scillitani et al., 2020). Ca^{2+} absorption is achieved through the active transport and passive diffusion (Duan et al., 2020). Its absorption is related to the Mg^{2+} presence, involving the ATP-dependent ionic pumps that transfer Ca^{2+} in the extracellular space exchanging calcium with Na^+ (Veklich et al., 2015). Mg^{2+} could be also indirectly influenced by Na^+ and K^+ concentrations since it is involved in the activity of the sodium-potassium pump (Rodrigues et al., 2021). Gastrointestinal system and kidneys closely regulate Mg^{2+} absorption and elimination (Al Alawi et al., 2021), and its intestinal active and passive uptake mechanisms seem to do not be under hormonal control (Vormann, 2003). For this reason, Mg^{2+} concentration could be considered a translational parameter to *in vivo,* that indicates the nutrients' bioaccessibility in the following perfusion model.

In line with the registered trend of Mg^{2+}, Ca^{2+} levels significantly raised in blood and dropped in the intestinal lumen content. Ca^{2+}, Mg^{2+} and K^+ plasma concentrations showed a decrease in case of enteritis *in vivo* which can impair the absorption functionality of the gut epithelium (Kimmel et al., 2000; Bhat et al., 2013). In our duodenum perfusion segment model, luminal and plasma mineral concentrations demonstrated that the bioaccessibility of these nutrients was maintained over three hours of extracorporeal circulation.

The lactate dehydrogenase is a cytosolic enzyme that catalyzes the conversion of lactate to pyruvate. LDH measurements offer a non-invasive and objective indication of mucosal cellular integrity since the extracellular localization of LDH is associated with an epithelial tissue injury (Sreenivasan et al., 2021). LDH blood concentrations did not show any difference over time even if higher numerical values were registered after 3 hours. During an organ injury LDH is released from dead cells and its concentration increases both in plasma and intestinal content (Mura et al., 2007). In addition, LDH is strongly correlated with hemoglobin release in blood vessels due to hemolysis (Sullivan and Faulds, 2013). The registered levels of LDH in plasma suggests that peristaltic pump and pressure conditions used for extracorporeal circulation were suitable in terms of erythrocytes integrity (Poder et al., 2017). The perfused intestinal segment model showed a progressive increase of luminal concentration of LDH suggesting a progressive damage of epithelial mucosa, consistent with the intestinal de-epithelialization and necrosis observed during the histological evaluation.

In this model, LDH concentrations in plasma and intestinal lumen content could be considered as reliable indirect control to assess organ viability over time.

In line with Sundaram et al. (2020), nitric oxide was not detectable, probably due to the limited ability of swine enterocytes to produce NO_2^-.

The preservation of tissue integrity before and during the experimentation is pivotal to ensure the accuracy and reproducibility of data (Sjöberg et al., 2013). Histological evaluation revealed that the overall intestinal architecture was preserved after 3 hours, with a visible blood perfusion and only a slight de-epithelialization of villi in portions A and B, while in portion C perfusion was absent and a diffuse loss of intestinal villi was observed. In this distal portion the nutrient absorption was likely impaired. Intestinal proliferation resulted highest in proximal portion A with a progressive decrease in B and C, supporting a reduced vitality in particular in the distal portion C. In the examined portions of duodenum, the observed enteritis was considered a spontaneous finding not unexpected in a farming pig. Preservation of epithelial structures for 3 hours was proposed as optimal period to optimize organ viability in *ex vivo* studies (Sutton et al., 2014). The observed increase in LDH was likely due to the de-epithelization of the intestinal mucosa. Hyperemia observed in the portions A and B was probably due to high pressure conditions, even if the selected parameters were in accordance with previous studies. In this model, pressure conditions require to be adapted in accordance with the size of the considered intestinal segment, and this aspect will require further adaptations in order to perfection this model. Literature studies demonstrated that UW solution provides better conservation of patches through prolonged ischemia compared to other solutions (de Sousa et al., 2021).

Further studies are needed to assess the glucose absorption trough perfusion of higher glucose concentrations through the intraluminal line. In addition, more data are required to investigate and optimize the luminal nutrition in order to maximize the organ viability and the conservation of intestinal structure for longer periods.

5. Conclusions

The developed swine perfused intestinal segment model showed characteristics of organ viability and functionality over three hours of extracorporeal circulation. The duodenum segment preserved the principal nutrients bioaccessibility, cellular metabolism, and, at histological evaluation, showed a preserved intestinal epithelial lining in the perfused portions. This study offered encouraging results for the development of a novel swine intestinal segment perfusion model for the evaluation of nutrients bioaccessibility in line with the 3R principle. Future studies will be useful to improve the organ viability and structures conservation, considering the potential of this model also for application to translational medicine for intestinal transplantations.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

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All authors drafted and approved the final version of the manuscript.

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Data Availability Statement

All data are available within the manuscript.

6. References

Abd El-Hack, M.E., El-Saadony, M.T., Elbestawy, A.R., Gado, A.R., Nader, M.M., Saad, A.M., El-Tahan, A.M., Taha, A.E., Salem, H.M., El-Tarabily, K.A., 2022. Hot red pepper powder as a safe alternative to antibiotics in organic poultry feed: An updated overview. Poultry Science, 101684.

Adhikari, P., Yadav, S., Cosby, D.E., Cox, N.A., Jendza, J.A., Kim, W.K., 2020. Research Note: Effect of organic acid mixture on growth performance and Salmonella Typhimurium colonization in broiler chickens. Poultry science 99, 2645-2649.

Aiyegoro, O., Dlamini, Z., Okoh, A., Langa1, R., 2017. Effects of probiotics on growth performance, blood parameters, and antibody stimulation in piglets. South African Journal of Animal Science 47, 766-775.

Al Alawi, A.M., Al Badi, A., Al Huraizi, A., Falhammar, H., 2021. Magnesium: The recent research and developments. Advances in Food and Nutrition Research 96, 193-218.

Al-Alo, K., Brujeni, G.N., Lotfollahzadeh, S., Moosakhani, F., Gharabaghi, A., 2018. Correlation between neonatal calf diarrhea and the level of maternally derived antibodies. Iranian Journal of Veterinary Research 19, 3.

Aland, A., Madec, F., 2009. Sustainable animal production: the challenges and potential developments for professional farming. Wageningen Academic Publishers.

Alayande, K.A., Aiyegoro, O.A., Ateba, C.N., 2020. Probiotics in animal husbandry: Applicability and associated risk factors. Sustainability 12, 1087.

Alexandre, F., Fabiani, J., 2010. Circolazione extracorporea. EMC-Tecniche Chirurgiche-Chirurgia Generale 10, 1-22.

Allen, H.K., Levine, U.Y., Looft, T., Bandrick, M., Casey, T.A., 2013. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. Trends in microbiology 21, 114-119.

Alshaibani, D., Zhang, R., Wu, V.C., 2017. Antibacterial characteristics and activity of Vaccinium macrocarpon proanthocyanidins against diarrheagenic Escherichia coli. Journal of Functional Foods 39, 133-138.

AlSheikh, H.M.A., Sultan, I., Kumar, V., Rather, I.A., Al-Sheikh, H., Tasleem Jan, A., Haq, Q.M.R., 2020. Plant-based phytochemicals as possible alternative to antibiotics in combating bacterial drug resistance. Antibiotics 9, 480.

Amalaradjou, M.A.R., Narayanan, A., Venkitanarayanan, K., 2011. Trans-cinnamaldehyde decreases attachment and invasion of uropathogenic Escherichia coli in urinary tract epithelial cells by modulating virulence gene expression. The Journal of urology 185, 1526-1531.

Amaral-Phillips, D.M., Scharko, P.B., Johns, J.T., Franklin, S., 2006. Feeding and managing baby calves from birth to 3 months of age. UK Cooperative Extension Service, University of Kentucky, ASC-161.
Amorim, A.B., Saleh, M.A.D., Miassi, G.d.M., Berto, D.A., 2018. Dietary supplementation with glutamine or glutamic acid for weanling piglets. Pesquisa Agropecuária Brasileira 53, 229-237.
AOAC, 2019. Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC.
Apak, R.a., Özyürek, M., Güçlü, K., Çapanoğlu, E., 2016. Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. Journal of agricultural and food chemistry 64, 997-1027.
Apás, A.L., Dupraz, J., Ross, R., González, S.N., Arena, M.E., 2010. Probiotic administration effect on fecal mutagenicity and microflora in the goat's gut. Journal of bioscience and bioengineering 110, 537-540.
Araujo, G., Bach, A., Mereu, A., Ipharraguerre, I., 2013. Effects of supplementing the milk replacer with tributyrin on the metabolism of Holstein calves. XV Jornadas sobre Producción Animal, Zaragoza 14 y 15 de mayo de 2013, 300-302.
Araujo, G., Terré, M., Mereu, A., Ipharraguerre, I., Bach, A., 2015. Effects of supplementing a milk replacer with sodium butyrate or tributyrin on performance and metabolism of Holstein calves. Animal Production Science 56, 1834-1841.
Arora, T., Mehta, A., Joshi, V., Mehta, K., Rathor, N., Mediratta, P., Sharma, K., 2011. Substitute of animals in drug research: an approach towards fulfillment of 4R's. Indian journal of pharmaceutical sciences 73, 1.
Arshad, M.A., Hassan, F.-u., Rehman, M.S., Huws, S.A., Cheng, Y., Din, A.U., 2021. Gut microbiome colonization and development in neonatal ruminants: Strategies, prospects, and opportunities. Animal Nutrition 7, 883-895.
Avelar Rodriguez, D., Peña Vélez, R., Toro Monjaraz, E.M., Ramirez Mayans, J., Ryan, P.M., 2019. The gut microbiota: a clinically impactful factor in patient health and disease. SN Comprehensive Clinical Medicine 1, 188-199.
Bag, A., Bhattacharyya, S., Chattopadhyay, R., 2013. Isolation and identification of a gallotannin 1, 2, 6-tri-O-galloyl-β-d-glucopyranose from hydroalcoholic extract of T erminalia chebula fruits effective against multidrug-resistant uropathogens. Journal of Applied Microbiology 115, 390-397.
Baghban-Kanani, P., Hosseintabar-Ghasemabad, B., Azimi-Youvalari, S., Seidavi, A., Ragni, M., Laudadio, V., Tufarelli, V., 2019. Effects of using Artemisia annua leaves, probiotic blend, and organic acids on performance, egg quality, blood biochemistry, and antioxidant status of laying hens. The Journal of Poultry Science 56, 120-127.
Baird, L., Dinkova-Kostova, A.T., 2011. The cytoprotective role of the Keap1–Nrf2 pathway. Archives of toxicology 85, 241-272.
Bajagai, Y.S., Klieve, A.V., Dart, P.J., Bryden, W.L., 2016. Probiotics in animal nutrition: production, impact and regulation. FAO.
Baloš, M.Ž., Jakšić, S., Knežević, S., Kapetanov, M., 2016. Electrolytes–sodium, potassium and chlorides in poultry nutrition. Archives of Veterinary Medicine 9, 31-42.
Barré-Sinoussi, F., Montagutelli, X., 2015. Animal models are essential to biological research: issues and perspectives. Future science OA 1.
Bartoš, P., Dolan, A., Smutný, L., Šístková, M., Celjak, I., Šoch, M., Havelka, Z., 2016. Effects of phytogenic feed additives on growth performance and on ammonia and greenhouse gases emissions in growing-finishing pigs. Animal Feed Science and Technology 212, 143-148.

Baydoun, M., Treizeibre, A., Follet, J., Vanneste, S., Creusy, C., Dercourt, L., Delaire, B., Mouray, A., Viscogliosi, E., Certad, G., Senez, V., 2020. An Interphase Microfluidic Culture System for the Study of Ex Vivo Intestinal Tissue. Micromachines 11.
Belkaid, Y., Hand, T.W., 2014. Role of the microbiota in immunity and inflammation. Cell 157, 121-141.
Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., Gil, A., 2012. Probiotic mechanisms of action. Annals of Nutrition and Metabolism 61, 160-174.
Berni Canani, R., Di Costanzo, M., Leone, L., 2012. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. Clinical epigenetics 4, 1-7.
Bernstein, N., Gorelick, J., Zerahia, R., Koch, S., 2019. Impact of N, P, K, and humic acid supplementation on the chemical profile of medical cannabis (Cannabis sativa L). Frontiers in plant science 10, 736.
Bhat, A.A., Wadhwa, D.R., Singh, S.P., Singh, I., 2013. Haematological and biochemical analysis in canine enteritis. Vet World 6, 380-383.
Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E.G., Mackie, R., Liu, J., Mu, C., Huang, R., Smidt, H., 2016. Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. Environmental microbiology 18, 1566-1577.
Binot, A., Duboz, R., Promburom, P., Phimpraphai, W., Cappelle, J., Lajaunie, C., Goutard, F.L., Pinyopummintr, T., Figuié, M., Roger, F.L., 2015. A framework to promote collective action within the One Health community of practice: using participatory modelling to enable interdisciplinary, cross-sectoral and multi-level integration. One Health 1, 44-48.
Biolley, C., Tretola, M., Bee, G., Jud, C., Silacci, P., 2019. Punicalagin increases glutamate absorption in differentiated Caco-2 cells by a mechanism involving gene expression regulation of an EAAT3 transporter. Food & function 10, 5333-5338.
Bogere, P., Choi, Y., Heo, J., 2019. Probiotics as alternatives to antibiotics in treating post-weaning diarrhoea in pigs. South African Journal of Animal Science 49, 403-416.
Borrelli, L., Varriale, L., Dipineto, L., Pace, A., Menna, L.F., Fioretti, A., 2021. Insect derived lauric acid as promising alternative strategy to antibiotics in the antimicrobial resistance scenario. Frontiers in Microbiology 12, 620798.
Bortoluzzi, C., Pedroso, A., Mallo, J., Puyalto, M., Kim, W., Applegate, T., 2017. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. Poultry science 96, 3981-3993.
Brewer, M., 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Comprehensive reviews in food science and food safety 10, 221-247.
Brunauer, M., Roch, F.-F., Conrady, B., 2021. Prevalence of worldwide neonatal calf diarrhoea caused by bovine rotavirus in combination with Bovine Coronavirus, Escherichia coli K99 and Cryptosporidium spp.: a meta-analysis. Animals 11, 1014.
Buntyn, J.O., Schmidt, T., Nisbet, D.J., Callaway, T.R., 2016. The role of direct-fed microbials in conventional livestock production.
Byrne, C., Chambers, E., Morrison, D., Frost, G., 2015. The role of short chain fatty acids in appetite regulation and energy homeostasis. International journal of obesity 39, 1331-1338.
Bédard, P., Gauvin, S., Ferland, K., Caneparo, C., Pellerin, È., Chabaud, S., Bolduc, S., 2020. Innovative human three-dimensional tissue-engineered models as an alternative to animal testing. Bioengineering 7, 115.

Cai, J., Chen, H., Weng, M., Jiang, S., Gao, J., 2019. Diagnostic and clinical significance of serum levels of D-lactate and diamine oxidase in patients with Crohn's disease. Gastroenterology Research and Practice 2019.
Cakmak, K.C., Gülçin, İ., 2019. Anticholinergic and antioxidant activities of usnic acid-An activity-structure insight. Toxicology reports 6, 1273-1280.
Canani, R.B., Di Costanzo, M., Leone, L., 2012. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. Clinical epigenetics 4, 1-7.
Canellas, L.P., Olivares, F.L., 2014. Physiological responses to humic substances as plant growth promoter. Chemical and Biological Technologies in Agriculture 1, 1-11.
Caprarulo, V., Giromini, C., Rossi, L., 2020. Chestnut and quebracho tannins in pig nutrition: the effects on performance and intestinal health. Animal, 100064.
Chan, B.C., Ip, M., Lau, C.B., Lui, S., Jolivalt, C., Ganem-Elbaz, C., Litaudon, M., Reiner, N.E., Gong, H., See, R.H., 2011. Synergistic effects of baicalein with ciprofloxacin against NorA over-expressed methicillin-resistant Staphylococcus aureus (MRSA) and inhibition of MRSA pyruvate kinase. Journal of ethnopharmacology 137, 767-773.
Chan, C.-L., Gan, R.-y., Shah, N.P., Corke, H., 2018a. Enhancing antioxidant capacity of Lactobacillus acidophilus-fermented milk fortified with pomegranate peel extracts. Food bioscience 26, 185-192.
Chan, C.-L., Gan, R.-Y., Shah, N.P., Corke, H., 2018b. Polyphenols from selected dietary spices and medicinal herbs differentially affect common food-borne pathogenic bacteria and lactic acid bacteria. Food Control 92, 437-443.
Chan, C.L., Gan, R.Y., Corke, H., 2016. The phenolic composition and antioxidant capacity of soluble and bound extracts in selected dietary spices and medicinal herbs. International Journal of Food Science & Technology 51, 565-573.
Chang, Q., Lu, Z., He, M., Gao, R., Bai, H., Shi, B., Shan, A., 2014. Effects of dietary supplementation of fulvic acid on lipid metabolism of finishing pigs. Journal of animal science 92, 4921-4926.
Chiang, M.-L., Chen, H.-C., Chen, K.-N., Lin, Y.-C., Lin, Y.-T., Chen, M.-J., 2015. Optimizing production of two potential probiotic Lactobacilli strains isolated from piglet feces as feed additives for weaned piglets. Asian-Australasian journal of animal sciences 28, 1163.
Chlebicz, A., Śliżewska, K., 2020. In vitro detoxification of aflatoxin B1, deoxynivalenol, fumonisins, T-2 toxin and zearalenone by probiotic bacteria from genus Lactobacillus and Saccharomyces cerevisiae yeast. Probiotics and antimicrobial proteins 12, 289-301.
Cho, Y.-i., Yoon, K.-J., 2014. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. Journal of veterinary science 15, 1-17.
Choi, Y., Goel, A., Hosseindoust, A., Lee, S., Kim, K., Jeon, S., Noh, H., Kyong Kwon, I., Chae, B., 2016. Effects of dietary supplementation of Ecklonia cava with or without probiotics on the growth performance, nutrient digestibility, immunity and intestinal health in weanling pigs. Italian Journal of Animal Science 15, 62-68.
Christaki, E., Florou-Paneri, P., Bonos, E., 2011. Microalgae: a novel ingredient in nutrition. International journal of food sciences and nutrition 62, 794-799.
Clara, R., Schumacher, M., Ramachandran, D., Fedele, S., Krieger, J.P., Langhans, W., Mansouri, A., 2017. Metabolic adaptation of the small intestine to short-and medium-term high-fat diet exposure. Journal of cellular physiology 232, 167-175.
Cormican, M., Hopkins, S., Jarlier, V., Reilly, J., Simonsen, G., Strauss, R., Vandenberg, O., Zabicka, D., Zarb, P., Catchpole, M., Heuer, O., Iosifidis, E., Monnet, D., Plachouras, D., Weist, K., Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Escamez, P., Girones, R.,

Herman, L., Koutsoumanis, K., Lindqvist, R., Norrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlstrom, H., Tenhagen, B., Teale, C., Schuepbach, G., Beloeil, P., Liebana, E., Stella, P., Murphy, D., Hauser, B., Urbain, B., Kozhuharov, E., Bozic, F., Michaelidou-Patsia, A., Bures, J., Vestergaard, E., Baptiste, K., Tiirats, T., Nevalainen, M., Rouby, J., Hahn, G., Schlumbohm, W., Malemis, I., Kulcsar, G., Lenhardsson, J., Beechinor, J., Breathnach, R., Pasquali, P., Auce, Z., Maciulskis, P., Schmit, M., Spiteri, S., Schefferlie, G., Hekman, P., Bergendahl, H., Swiezcicka, A., Da Silva, J., Taban, L., Hederova, J., Straus, K., Madero, C., Persson, E., Jukes, H., Weeks, J., Kivilahti-Mantyla, K., Moulin, G., Wallmann, J., Grave, K., Greko, C., Munoz, C., Bouchard, D., Catry, B., Moreno, M., Pomba, C., Rantala, M., Ruzauskas, M., Sanders, P., Schwarz, C., van Duijkeren, E., Wester, A., Ignate, K., Kunsagi, Z., Torren-Edo, J., ECDC, BIOH, E.P.B.H., CVMP, E.C.M.P.V., 2017. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. Efsa Journal 15.

Correddu, F., Lunesu, M.F., Buffa, G., Atzori, A.S., Nudda, A., Battacone, G., Pulina, G., 2020. Can agro-industrial by-products rich in polyphenols be advantageously used in the feeding and nutrition of dairy small ruminants? Animals 10, 131.

Costa, L., Luciano, F., Miyada, V., Gois, F., 2013. Herbal extracts and organic acids as natural feed additives in pig diets. South African Journal of Animal Science 43, 181-193.

Council, N.R., 2001. Nutrient requirements of dairy cattle: 2001. National Academies Press.

Cresci, G.A., Glueck, B., McMullen, M.R., Xin, W., Allende, D., Nagy, L.E., 2017. Prophylactic tributyrin treatment mitigates chronic-binge ethanol-induced intestinal barrier and liver injury. Journal of gastroenterology and hepatology 32, 1587-1597.

Croset, M., Rajas, F., Zitoun, C., Hurot, J.-M., Montano, S., Mithieux, G., 2001. Rat small intestine is an insulin-sensitive gluconeogenic organ. Diabetes 50, 740-746.

Dafale, N.A., Srivastava, S., Purohit, H.J., 2020. Zoonosis: an emerging link to antibiotic resistance under "one health approach". Indian journal of microbiology 60, 139-152.

de Melo, B.A.G., Motta, F.L., Santana, M.H.A., 2016. Humic acids: Structural properties and multiple functionalities for novel technological developments. Materials Science and Engineering: C 62, 967-974.

de Sousa, S.G., Nascimento da Silva, G.V., AM, C.R., da Silva, M.F., Costa, F.C., Santana de Macedo, B., Brito, M., 2021. Organ Preservation Solutions in Transplantation: A Literature Review. Experimental and Clinical Transplantation: Official Journal of the Middle East Society for Organ Transplantation.

De Vries, H., Smidt, H., 2020. Microbiota development in piglets. The Suckling and Weaned Piglet; Farmer, C., Ed.; Wageningen Academic Publishers: Wageningen, The Netherlands, 179-205.

Dell'Anno, M., Callegari, M.L., Reggi, S., Caprarulo, V., Giromini, C., Spalletta, A., Coranelli, S., Rossi, C.A.S., Rossi, L., 2021a. Lactobacillus plantarum and Lactobacillus reuteri as Functional Feed Additives to Prevent Diarrhoea in Weaned Piglets. Animals 11, 1766.

Dell'Anno, M., Reggi, S., Caprarulo, V., Hejna, M., Sgoifo Rossi, C.A., Callegari, M.L., Baldi, A., Rossi, L., 2021b. Evaluation of Tannin Extracts, Leonardite and Tributyrin Supplementation on Diarrhoea Incidence and Gut Microbiota of Weaned Piglets. Animals 11, 1693.

Demetrius, L., 2006. Aging in mouse and human systems: a comparative study. Annals of the New York Academy of Sciences 1067, 66-82.

Devle, H., Ulleberg, E.K., Naess-Andresen, C.F., Rukke, E.-O., Vegarud, G., Ekeberg, D., 2014. Reciprocal interacting effects of proteins and lipids during ex vivo digestion of bovine milk. International Dairy Journal 36, 6-13.
Dir. 2010/63/EU, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance.
Dittoe, D.K., Ricke, S.C., Kiess, A.S., 2018. Organic acids and potential for modifying the avian gastrointestinal tract and reducing pathogens and disease. Frontiers in veterinary science 5, 216.
Domínguez Díaz, L., Fernández-Ruiz, V., Cámara, M., 2020. The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. Critical Reviews in Food Science and Nutrition 60, 1738-1746.
Dong, G., Liu, H., Yu, X., Zhang, X., Lu, H., Zhou, T., Cao, J., 2018. Antimicrobial and anti-biofilm activity of tannic acid against Staphylococcus aureus. Natural product research 32, 2225-2228.
Dong, L., Zhong, X., He, J., Zhang, L., Bai, K., Xu, W., Wang, T., Huang, X., 2016. Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets. Clinical nutrition 35, 399-407.
Doyon, M., Labrecque, J., 2008. Functional foods: a conceptual definition. British Food Journal.
Du, K., Bereswill, S., Heimesaat, M.M., 2021. A literature survey on antimicrobial and immune-modulatory effects of butyrate revealing non-antibiotic approaches to tackle bacterial infections. European Journal of Microbiology and Immunology 11, 1-9.
Duan, P., Yuan, X., Gan, S., Zhang, Y., Duan, W., 2020. Time delay induces saw-tooth calcium wave and transition in intracellular calcium oscillation. Chinese Journal of Physics 66, 150-156.
Duarte, M.E., Kim, S.W., 2022. Intestinal microbiota and its interaction to intestinal health in nursery pigs. Animal Nutrition 8, 169-184.
Dutot, M., Fagon, R., Hemon, M., Rat, P., 2012. Antioxidant, anti-inflammatory, and anti-senescence activities of a phlorotannin-rich natural extract from brown seaweed Ascophyllum nodosum. Applied biochemistry and biotechnology 167, 2234-2240.
Eberl, G., 2010. A new vision of immunity: homeostasis of the superorganism. Mucosal immunology 3, 450-460.
Ebrahimnejad, H., Burkholz, T., Jacob, C., 2014. Flavanols and proanthocyanidins, Recent advances in redox active plant and microbial products, Springer, pp. 211-232.
Eibl, C., Bexiga, R., Viora, L., Guyot, H., Félix, J., Wilms, J., Tichy, A., Hund, A., 2021. The Antibiotic Treatment of Calf Diarrhea in Four European Countries: A Survey. Antibiotics 10, 910.
Ekambaram, S.P., Perumal, S.S., Balakrishnan, A., 2016. Scope of hydrolysable tannins as possible antimicrobial agent. Phytotherapy Research 30, 1035-1045.
El Kolli, M., Laouer, H., El Kolli, H., Akkal, S., Sahli, F., 2016. Chemical analysis, antimicrobial and anti-oxidative properties of Daucus gracilis essential oil and its mechanism of action. Asian Pacific Journal of Tropical Biomedicine 6, 8-15.
El-Tawab, A., Youssef, I., Bakr, H., Fthenakis, G., Giadinis, N., 2016. Role of probiotics in nutrition and health of small ruminants. Polish Journal of Veterinary Sciences.
Elmastas, M., Celik, S.M., Genc, N., Aksit, H., Erenler, R., Gulcin, İ., 2018. Antioxidant activity of an Anatolian herbal tea—Origanum minutiflorum: isolation and characterization of its secondary metabolites. International journal of food properties 21, 374-384.

EMA, CVMP, CHMP, 2019. Categorisation of Antibiotics in the European Union. Answer to the Request from the European Commission for Updating the Scientific Advice on the Impact on Public Health and Animal Health of the Use of Antibiotics in Animals, European Medicines Agency Amsterdam, The Netherlands.
Emami, N.K., Daneshmand, A., Naeini, S.Z., Graystone, E., Broom, L., 2017. Effects of commercial organic acid blends on male broilers challenged with E. coli K88: Performance, microbiology, intestinal morphology, and immune response. Poultry science 96, 3254-3263.
Engels, C., Schieber, A., Gänzle, M.G., 2011. Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (Mangifera indica L.). Applied and Environmental Microbiology 77, 2215-2223.
Enzing, C., Ploeg, M., Barbosa, M., Sijtsma, L., 2014. Microalgae-based products for the food and feed sector: an outlook for Europe. JRC Scientific and policy reports, 19-37.
Erickson, P.S., Marston, S., Gemmel, M., Deming, J., Cabral, R., Murphy, M., Marden, J., 2012. Kelp taste preferences by dairy calves. Journal of dairy science 95, 856-858.
Ericsson, A.C., Crim, M.J., Franklin, C.L., 2013. A brief history of animal modeling. Missouri medicine 110, 201.
Etienne-Mesmin, L., Chassaing, B., Desvaux, M., De Paepe, K., Gresse, R., Sauvaitre, T., Forano, E., de Wiele, T.V., Schüller, S., Juge, N., 2019. Experimental models to study intestinal microbes–mucus interactions in health and disease. FEMS microbiology reviews 43, 457-489.
European Food Safety Authority (EFSA), 2015. EFSA Strategy 2020 Trusted science for safe food.
Evans, F., Critchley, A., 2014. Seaweeds for animal production use. Journal of applied phycology 26, 891-899.
Everaert, N., Van Cruchten, S., Weström, B., Bailey, M., Van Ginneken, C., Thymann, T., Pieper, R., 2017. A review on early gut maturation and colonization in pigs, including biological and dietary factors affecting gut homeostasis. Animal feed science and technology 233, 89-103.
FAO, 2018. World Livestock: Transforming the livestock sector through the Sustainable Development Goals. , Rome, p. 222 pp.
Farha, A.K., Yang, Q.-Q., Kim, G., Li, H.-B., Zhu, F., Liu, H.-Y., Gan, R.-Y., Corke, H., 2020. Tannins as an alternative to antibiotics. Food Bioscience 38, 100751.
FEFAC, 2020. The European Feed Manufacturers' Federation. Feed Sustainability Charter 2030.
Ferdous, U.T., Yusof, Z.N.B., 2021. Medicinal prospects of antioxidants from algal sources in cancer therapy. Frontiers in Pharmacology 12, 593116.
Foditsch, C., Pereira, R.V.V., Ganda, E.K., Gomez, M.S., Marques, E.C., Santin, T., Bicalho, R.C., 2015. Oral administration of Faecalibacterium prausnitzii decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers. PloS one 10, e0145485.
Food and Agricolture Organization (FAO), 2017. The future of food and agriculture-Trends and challenges. Rome, Italy.
Frazzini, S., Scaglia, E., Dell'Anno, M., Reggi, S., Panseri, S., Giromini, C., Lanzoni, D., Sgoifo Rossi, C.A., Rossi, L., 2022. Antioxidant and Antimicrobial Activity of Algal and Cyanobacterial Extracts: An In Vitro Study. Antioxidants 11, 992.
Frese, S.A., Parker, K., Calvert, C.C., Mills, D.A., 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3, 1-10.

Frieten, D., Gerbert, C., Koch, C., Dusel, G., Eder, K., Kanitz, E., Weitzel, J., Hammon, H., 2017. Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves. Journal of dairy science 100, 6648-6661.
Fukuda, T., Tsukano, K., Nakatsuji, H., Suzuki, K., 2019. Plasma diamine oxidase activity decline with diarrhea severity in calves indicating systemic dysfunction related to intestinal mucosal damage. Research in Veterinary Science 126, 127-130.
Gadde, U., Kim, W., Oh, S., Lillehoj, H.S., 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Animal health research reviews 18, 26-45.
Gahan, D., Lynch, M., Callan, J., O'sullivan, J., O'Doherty, J., 2009. Performance of weanling piglets offered low-, medium-or high-lactose diets supplemented with a seaweed extract from Laminaria spp. Animal 3, 24-31.
Galley, J.D., Yu, Z., Kumar, P., Dowd, S.E., Lyte, M., Bailey, M.T., 2014. The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor. Gut microbes 5, 748-760.
Geletu, U.S., Usmael, M.A., Bari, F.D., 2021. Rotavirus in calves and its zoonotic importance. Veterinary Medicine International 2021.
Gemeda, N., Tadele, A., Lemma, H., Girma, B., Addis, G., Tesfaye, B., Abebe, A., Gemechu, W., Yirsaw, K., Teka, F., 2018. Development, characterization, and evaluation of novel broad-spectrum antimicrobial topical formulations from Cymbopogon martini (Roxb.) W. Watson essential oil. Evidence-Based Complementary and Alternative Medicine 2018.
Giacchetti, G., Sechi, L.A., Rilli, S., Carey, R.M., 2005. The renin–angiotensin–aldosterone system, glucose metabolism and diabetes. Trends in Endocrinology & Metabolism 16, 120-126.
Gil-Gil, T., Ochoa-Sánchez, L.E., Baquero, F., Martínez, J.L., 2021. Antibiotic resistance: time of synthesis in a post-genomic age. Computational and Structural Biotechnology Journal 19, 3110-3124.
González-Ríos, H., Dávila-Ramírez, J., Peña-Ramos, E., Valenzuela-Melendres, M., Zamorano-García, L., Islava-Lagarda, T., Valenzuela-Grijalva, N., 2016. Dietary supplementation of ferulic acid to steers under commercial feedlot feeding conditions improves meat quality and shelf life. Animal Feed Science and Technology 222, 111-121.
Gouveia, L., Oliveira, A.C., 2009. Microalgae as a raw material for biofuels production. Journal of industrial microbiology and biotechnology 36, 269-274.
Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L.D., Hidalgo, F.J., Miyashita, K., van Camp, J., Alasalvar, C., Ismail, A.B., 2018. Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? Food chemistry 264, 471-475.
Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E., Blanquet-Diot, S., 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends in microbiology 25, 851-873.
Griffiths, N.M., Van der Meeren, A., Angulo, J.F., Vincent-Naulleau, S., 2020. Research on the Radiotoxicology of Plutonium Using Animals: Consideration of the 3Rs—: Replace, Reduce, Refine. Health physics 119, 133-140.
Grossi, S., Compiani, R., Baldi, G., Rossi, C.A.S., 2021a. The Effect of Yucca Schidigera inclusion in milk replacer for veal calves on health status, antimicrobial use and growth performance. Large Animal Review 27, 251-257.
Grossi, S., Dell'Anno, M., Rossi, L., Compiani, R., Sgoifo Rossi, C.A., 2021b. Supplementation of Live Yeast, Mannan Oligosaccharide, and Organic Selenium during the Adaptation Phase

of Newly Arrived Beef Cattle: Effects on Health Status, Immune Functionality, and Growth Performance. Antibiotics 10, 1114.
Gröber, U., Schmidt, J., Kisters, K., 2015. Magnesium in prevention and therapy. Nutrients 7, 8199-8226.
Guibert, E.E., Petrenko, A.Y., Balaban, C.L., Somov, A.Y., Rodriguez, J.V., Fuller, B.J., 2011. Organ preservation: current concepts and new strategies for the next decade. Transfusion Medicine and Hemotherapy 38, 125-142.
Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., Van Immerseel, F., 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutrition research reviews 23, 366-384.
Guilloteau, P., Zabielski, R., David, J.-C., Blum, J.W., Morisset, J.A., Biernat, M., Woliński, J., Laubitz, D., Hamon, Y., 2009. Sodium-butyrate as a growth promoter in milk replacer formula for young calves. Journal of dairy science 92, 1038-1049.
Gulcin, İ., 2020. Antioxidants and antioxidant methods: An updated overview. Archives of toxicology 94, 651-715.
Gupta, A., Dwivedi, M., Mahdi, A.A., Nagana Gowda, G., Khetrapal, C.L., Bhandari, M., 2012. Inhibition of adherence of multi-drug resistant E. coli by proanthocyanidin. Urological research 40, 143-150.
Gutzeit, C., Magri, G., Cerutti, A., 2014. Intestinal IgA production and its role in host-microbe interaction. Immunological reviews 260, 76-85.
Górka, P., Kowalski, Z., Zabielski, R., Guilloteau, P., 2018. Invited review: Use of butyrate to promote gastrointestinal tract development in calves. Journal of dairy science 101, 4785-4800.
Gülcin, I., 2012. Antioxidant activity of food constituents: an overview. Archives of toxicology 86, 345-391.
Hamed, M., Barlow, A., Dolezalova, N., Khosla, S., Sagar, A., Gribble, F., Davies, S., Murphy, M., Hosgood, S., Nicholson, M., 2021. Ex vivo normothermic perfusion of isolated segmental porcine bowel: a novel functional model of the small intestine. BJS open 5, zrab009.
Hanczakowska, E., Świątkiewicz, M., Natonek-Wiśniewska, M., Okoń, K., 2016. Medium chain fatty acids (MCFA) and/or probiotic Enterococcus faecium as a feed supplement for piglets. Livestock science 192, 1-7.
Hansen, V.L., Kahl, S., Proszkowiec-Weglarz, M., Jiménez, S.C., Vaessen, S.F., Schreier, L.L., Jenkins, M.C., Russell, B., Miska, K.B., 2021. The effects of tributyrin supplementation on weight gain and intestinal gene expression in broiler chickens during Eimeria maxima-induced coccidiosis. Poultry Science 100, 100984.
Hatew, B., Inabu, Y., Sugino, T., Steele, M., 2019. Effects of pulse-dose ruminal infusion of butyrate on plasma glucagon-like peptide 1 and 2 concentrations in dairy calves. Journal of dairy science 102, 2254-2265.
Heinritz, S.N., Mosenthin, R., Weiss, E., 2013. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. Nutrition research reviews 26, 191-209.
Heo, J.M., Opapeju, F., Pluske, J., Kim, J., Hampson, D., Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. Journal of animal physiology and animal nutrition 97, 207-237.
Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews Gastroenterology & hepatology.

Hill, T., Aldrich, J., Schlotterbeck, R., Bateman II, H., 2007. Effects of changing the fat and fatty acid composition of milk replacers fed to neonatal calves. The Professional Animal Scientist 23, 135-143.
Honda, K., Littman, D.R., 2016. The microbiota in adaptive immune homeostasis and disease. Nature 535, 75-84.
Hotel, A.C.P., Cordoba, A., 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Prevention 5, 1-10.
Hou, Y., Wang, L., Yi, D., Ding, B., Chen, X., Wang, Q., Zhu, H., Liu, Y., Yin, Y., Gong, J., Wu, G., 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. British Journal of Nutrition 111, 1748-1758.
Hu, Q., Yin, F., Yang, L., Li, B., Lei, G., Wang, C., Yin, Y., Liu, D., 2022. Dietary tributyrin intervention improves the carcass traits, organ indices, and blood biomarker profiles in broilers under the isocaloric diets administration. Poultry Science, 102061.
Huang, C., Song, P., Fan, P., Hou, C., Thacker, P., Ma, X., 2015. Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. The Journal of nutrition 145, 2774-2780.
Hull, M.A., 2021. Nutritional prevention of colorectal cancer. Proceedings of the Nutrition Society 80, 59-64.
Humann, J., Lenz, L.L., 2009. Bacterial peptidoglycan-degrading enzymes and their impact on host muropeptide detection. Journal of innate immunity 1, 88-97.
Hyams, C., Camberlein, E., Cohen, J.M., Bax, K., Brown, J.S., 2010. The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infection and immunity 78, 704-715.
Inabu, Y., Murayama, K., Inouchi, K., Sugino, T., 2019. The effect of tributyrin supplementation to milk replacer on plasma glucagon-like peptide 2 concentrations in pre-weaning calves. Animal Science Journal 90, 1185-1192.
Ipek, H., Avci, M., Iriadam, M., Kaplan, O., Denek, N., 2008. Effects of humic acid on some hematological parameters, total antioxidant capacity and laying performance in Japanese quails. Archiv fur Geflugelkunde 72, 56-60.
Islam, K.M.S., Schuhmacher, A., Gropp, J.M., 2005. Humic Acid Substances in Animal Agriculture. Pakistan Journal of Nutrition 4, 126-134.
Jiang, W., Sunkara, L.T., Zeng, X., Deng, Z., Myers, S.M., Zhang, G., 2013. Differential regulation of human cathelicidin LL-37 by free fatty acids and their analogs. Peptides 50, 129-138.
Jin, I.S., Yoon, M.S., Park, C.-W., Hong, J.T., Chung, Y.B., Kim, J.-S., Shin, D.H., 2020. Replacement techniques to reduce animal experiments in drug and nanoparticle development. Journal of Pharmaceutical Investigation 50, 327-335.
Kalin, P., Gülçin, İ., Gören, A.C., 2015. Antioxidant activity and polyphenol content of cranberries (Vaccinium macrocarpon). Records of Natural Products 9, 496.
Karamzadeh-Dehaghani, A., Towhidi, A., Zhandi, M., Mojgani, N., Fouladi-Nashta, A., 2021. Combined effect of probiotics and specific immunoglobulin Y directed against Escherichia coli on growth performance, diarrhea incidence, and immune system in calves. Animal 15, 100124.
Khan, A., Pessin, J., 2002. Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. Diabetologia 45, 1475-1483.

Khan, D., Vasu, S., Moffett, R.C., Irwin, N., Flatt, P.R., 2017. Differential expression of glucagon-like peptide-2 (GLP-2) is involved in pancreatic islet cell adaptations to stress and beta-cell survival. Peptides 95, 68-75.
Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial cell factories 17, 1-21.
Khan, R.U., Chand, N., Akbar, A., 2016. Effect of organic acids on the performance of Japanese quails. Pakistan Journal of Zoology 48.
Khan, R.U., Naz, S., Raziq, F., Qudratullah, Q., Khan, N.A., Laudadio, V., Tufarelli, V., Ragni, M., 2022. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environmental Science and Pollution Research, 1-11.
Khanbabaee, K., Van Ree, T., 2001. Tannins: classification and definition. Natural product reports 18, 641-649.
Khawaskar, D.P., Sinha, D., Lalrinzuala, M.V., Athira, V., Kumar, M., Chhakchhuak, L., Mohanapriya, K., Sophia, I., Kumar, O., Chaudhuri, P., 2021. Pathotyping and antimicrobial susceptibility testing of Escherichia coli isolates from neonatal calves. Veterinary research communications, 1-10.
Khil'ko, S., Efimova, I., Smirnova, O., 2011. Antioxidant properties of humic acids from brown coal. Solid Fuel Chemistry 45, 367-371.
Kiarie, E., Romero, L.F., Nyachoti, C.M., 2013. The role of added feed enzymes in promoting gut health in swine and poultry. Nutrition research reviews 26, 71-88.
Kim, J.E., Lillehoj, H.S., Hong, Y.H., Kim, G.B., Lee, S.H., Lillehoj, E.P., Bravo, D.M., 2015. Dietary Capsicum and Curcuma longa oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. Research in veterinary science 102, 150-158.
Kim, S.-K., Himaya, S., 2011. Medicinal effects of phlorotannins from marine brown algae. Advances in food and nutrition research 64, 97-109.
Kimmel, S.E., Waddell, L.S., Michel, K.E., 2000. Hypomagnesemia and hypocalcemia associated with protein-losing enteropathy in Yorkshire terriers: five cases (1992–1998). Journal of the American Veterinary Medical Association 217, 703-706.
Knudsen, K., Laerke, H., Hedemann, M., 2018. Impact of dietmodulated butyrate production on intestinal barrier function and inflammation., Nutrients, p. 1499.
Kogut, M.H., 2019. The effect of microbiome modulation on the intestinal health of poultry. Animal feed science and technology 250, 32-40.
Kolodziejczyk, A.A., Zheng, D., Elinav, E., 2019. Diet–microbiota interactions and personalized nutrition. Nature Reviews Microbiology 17, 742-753.
Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Bäckhed, H.K., Gonzalez, A., Werner, J.J., Angenent, L.T., Knight, R., 2012. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 150, 470-480.
Kovanda, L., Zhang, W., Wei, X., Luo, J., Wu, X., Atwill, E.R., Vaessen, S., Li, X., Liu, Y., 2019. In vitro antimicrobial activities of organic acids and their derivatives on several species of gram-negative and gram-positive bacteria. Molecules 24, 3770.
Kumar, P., Chand, S., Maurya, P.K., 2016. Quercetin-modulated erythrocyte membrane sodium-hydrogen exchanger during human aging: correlation with ATPase's. Archives of physiology and biochemistry 122, 141-147.
Kyaw, B.M., Arora, S., Lim, C.S., 2011. Anti-Pseudomonal and anti-biofilm activity of tannic acid in combination with antibiotics. International Journal of Integrative Biology 11, 110.

Lallès, J.-P., Montoya, C.A., 2021. Dietary alternatives to in-feed antibiotics, gut barrier function and inflammation in piglets post-weaning: Where are we now? Animal Feed Science and Technology, 114836.
Lauridsen, C., Højberg, O., Kongsted, H., Canibe, N., 2017. A critical review on alternatives to antibiotics and pharmacological zinc for prevention of diarrhoea in pigs post-weaning. Rapport og vidensyntese, DCA 26.
Lauronen, J., Pakarinen, M.P., Pirinen, P., Kuusanmãki, P., Raivio, P., Savilahti, E., Paavonen, T., Halttunen, J., 2001. Effects of extrinsic denervation with or without ischemia–reperfusion injury on constitutional mucosal characteristics in porcine jejunoileum. Digestive diseases and sciences 46, 476-485.
Le, C.-F., Fang, C.-M., Sekaran, S.D., 2017. Intracellular targeting mechanisms by antimicrobial peptides. Antimicrobial agents and chemotherapy 61, e02340-02316.
Lee, M., Lin, W., Yu, B., Lee, T., 2017. Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals—A review. Asian-Australasian journal of animal sciences 30, 299.
Leonel, A.J., Teixeira, L.G., Oliveira, R.P., Santiago, A.F., Batista, N.V., Ferreira, T.R., Santos, R.C., Cardoso, V.N., Cara, D.C., Faria, A.M., 2013. Antioxidative and immunomodulatory effects of tributyrin supplementation on experimental colitis. British Journal of Nutrition 109, 1396-1407.
Lewis, K., Ausubel, F.M., 2006. Prospects for plant-derived antibacterials. Nature biotechnology 24, 1504-1507.
Li, F.-C., Li, Y.-K., Fan, Y.-C., Wang, K., 2018. Plasma concentration of diamine oxidase (DAO) predicts 1-month mortality of acute-on-chronic hepatitis B liver failure. Clinica Chimica Acta 484, 164-170.
Li, Y., Schilling, T., Benisch, P., Zeck, S., Meissner-Weigl, J., Schneider, D., Limbert, C., Seufert, J., Kassem, M., Schutze, N., Jakob, F., Ebert, R., 2007. Effects of high glucose on mesenchymal stem cell proliferation and differentiation. Biochemical and Biophysical Research Communications 363, 209-215.
Lillehoj, H., Liu, Y., Calsamiglia, S., Fernandez-Miyakawa, M., Chi, F., Cravens, R., Oh, S., Gay, C., 2018. Phytochemicals as antibiotic alternatives to promote growth and enhance host health. Veterinary Research 49.
Liu, J.-B., Ding, Y.-S., Zhang, Y., Chen, J.-B., Cui, B.-S., Bai, J.-Y., Lin, M.-B., Hou, Q., Zhang, P.-C., Li, S., 2015a. Anti-inflammatory hydrolyzable tannins from Myricaria bracteata. Journal of Natural Products 78, 1015-1025.
Liu, S., Chen, R., Hagedorn, C.H., 2015b. Tannic acid inhibits hepatitis C virus entry into Huh7. 5 cells. PLoS One 10, e0131358.
Liu, S., Wu, J., Wu, Z., Alugongo, G.M., Khan, M.Z., Li, J., Xiao, J., He, Z., Ma, Y., Li, S., 2022. Tributyrin administration improves intestinal development and health in pre-weaned dairy calves fed milk replacer. Animal Nutrition 10, 399-411.
Liu, W., La, A., Evans, A., Gao, S., Yu, Z., Bu, D., Ma, L., 2021. Supplementation with sodium butyrate improves growth and antioxidant function in dairy calves before weaning. Journal of Animal Science and Biotechnology 12, 1-9.
Liu, Y., Espinosa, C.D., Abelilla, J.J., Casas, G.A., Lagos, L.V., Lee, S.A., Kwon, W.B., Mathai, J.K., Navarro, D.M., Jaworski, N.W., 2018. Non-antibiotic feed additives in diets for pigs: a review. Animal Nutrition 4, 113-125.
Lo Verso, L., Talbot, G., Morissette, B., Guay, F., Matte, J.J., Farmer, C., Gong, J., Wang, Q., Bissonnette, N., Beaulieu, C., 2020. The combination of nutraceuticals and functional feeds as

additives modulates gut microbiota and blood markers associated with immune response and health in weanling piglets. Journal of animal science 98, skaa208.
Lombardi, P., Musco, N., Calabrò, S., Tudisco, R., Mastellone, V., Vastolo, A., Infascelli, F., Cutrignelli, M.I., 2020. Different carbohydrate sources affect swine performance and post-prandial glycaemic response. Italian Journal of Animal Science 19, 421-430.
Lopreiato, V., Mezzetti, M., Cattaneo, L., Ferronato, G., Minuti, A., Trevisi, E., 2020. Role of nutraceuticals during the transition period of dairy cows: A review. Journal of Animal Science and Biotechnology 11, 1-18.
Lorenzi, V., Muselli, A., Bernardini, A.F., Berti, L., Pagès, J.-M., Amaral, L., Bolla, J.-M., 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species. Antimicrobial agents and chemotherapy 53, 2209-2211.
Lueangsakulthai, J., Sah, B., Scottoline, B., Dallas, D., 2020. Survival of Recombinant Monoclonal Antibodies (IgG, IgA and sIgA) Versus Naturally-Occurring Antibodies (IgG and sIgA/IgA) in an Ex Vivo Infant Digestion Model. Nutrients 12.
Luise, D., Lauridsen, C., Bosi, P., Trevisi, P., 2019. Methodology and application of Escherichia coli F4 and F18 encoding infection models in post-weaning pigs. Journal of Animal Science and Biotechnology 10.
Lynegaard, J.C., Kjeldsen, N.J., Hansen, C.F., Williams, A.R., Nielsen, J.P., Amdi, C., 2022. Reduction in Diarrhoea and Modulation of Intestinal Gene Expression in Pigs Allocated a Low Protein Diet without Medicinal Zinc Oxide Post-Weaning. Animals 12, 989.
López-Andrés, P., Luciano, G., Vasta, V., Gibson, T.M., Biondi, L., Priolo, A., Mueller-Harvey, I., 2013. Dietary quebracho tannins are not absorbed, but increase the antioxidant capacity of liver and plasma in sheep. British Journal of Nutrition 110, 632-639.
Ma, N., Guo, P., Zhang, J., He, T., Kim, S.W., Zhang, G., Ma, X., 2018. Nutrients mediate intestinal bacteria–mucosal immune crosstalk. Frontiers in Immunology 9, 5.
Macit, M., Celebi, S., Esenbuga, N., Karaca, H., 2009. Effects of dietary humate supplementation on performance, egg quality and egg yolk fatty acid composition in layers. Journal of the Science of Food and Agriculture 89, 315-319.
MacIver, N.J., Jacobs, S.R., Wieman, H.L., Wofford, J.A., Coloff, J.L., Rathmell, J.C., 2008. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. Journal of leukocyte biology 84, 949-957.
Mackenzie, J.S., Jeggo, M., 2019. The One Health approach—Why is it so important?, MDPI, p. 88.
Madeira, M.S., Cardoso, C., Lopes, P.A., Coelho, D., Afonso, C., Bandarra, N.M., Prates, J.A., 2017. Microalgae as feed ingredients for livestock production and meat quality: A review. Livestock Science 205, 111-121.
Maier, G.U., Breitenbuecher, J., Gomez, J.P., Samah, F., Fausak, E., Van Noord, M., 2022. Vaccination for the prevention of neonatal calf diarrhea in cow-calf operations: a scoping review. Veterinary and animal science, 100238.
Majumder, S., Sigamani, A., Rahman, T., Rahmatullah, M., 2020. Exploring phytochemicals as alternatives to antimicrobials-Prospects and potentials. World J. Pharm. Pharm. Sci 9, 1802-1813.
Malmuthuge, N., 2017. Understanding the gut microbiome of dairy calves: Opportunities to improve early-life gut health. Journal of Dairy Science 100, 5996-6005.
Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews 14, 217-232.

Mayer, M., Abenthum, A., Matthes, J., Kleeberger, D., Ege, M., Hölzel, C., Bauer, J., Schwaiger, K., 2012. Development and genetic influence of the rectal bacterial flora of newborn calves. Veterinary microbiology 161, 179-185.
McCall, J., Hidalgo, G., Asadishad, B., Tufenkji, N., 2013. Cranberry impairs selected behaviors essential for virulence in Proteus mirabilis HI4320. Canadian journal of microbiology 59, 430-436.
McDonnell, P., Figat, S., O'doherty, J., 2010. The effect of dietary laminarin and fucoidan in the diet of the weanling piglet on performance, selected faecal microbial populations and volatile fatty acid concentrations. Animal 4, 579-585.
McEwen, S.A., Collignon, P.J., 2018. Antimicrobial resistance: a one health perspective. Microbiology spectrum 6, 6.2. 10.
Meigs, L., Smirnova, L., Rovida, C., Leist, M., Hartung, T., 2018. Animal testing and its alternatives–The most important omics is economics. ALTEX-Alternatives to animal experimentation 35, 275-305.
Menichetti, B., Garcia-Guerra, A., Lakritz, J., Weiss, W., Velez, J., Bothe, H., Merchan, D., Schuenemann, G., 2021. Effect of timing of prepartum vaccination relative to pen change with an acidogenic diet on lying time and metabolic profile in Holstein dairy cows. Journal of Dairy Science 104, 11059-11071.
Mignacca, S.A., Dore, S., Spuria, L., Zanghì, P., Amato, B., Duprè, I., Armas, F., Biasibetti, E., Camperio, C., Lollai, S.A., 2017. Intramammary infusion of a live culture of Lactococcus lactis in ewes to treat staphylococcal mastitis. Journal of medical microbiology 66, 1798-1810.
Miragoli, F., Patrone, V., Prandini, A., Sigolo, S., Dell'Anno, M., Rossi, L., Senizza, A., Morelli, L., Callegari, M.L., 2021. Implications of Tributyrin on Gut Microbiota Shifts Related to Performances of Weaning Piglets. Microorganisms 9, 584.
Mishra, A., Sharma, A.K., Kumar, S., Saxena, A.K., Pandey, A.K., 2013. Bauhinia variegata leaf extracts exhibit considerable antibacterial, antioxidant, and anticancer activities. BioMed Research International 2013.
Miyoshi, M., Sakaki, H., Usami, M., Iizuka, N., Shuno, K., Aoyama, M., Usami, Y., 2011. Oral administration of tributyrin increases concentration of butyrate in the portal vein and prevents lipopolysaccharide-induced liver injury in rats. Clinical nutrition 30, 252-258.
Moita, V.H.C., Duarte, M.E., da Silva, S.N., Kim, S.W., 2021. Supplemental effects of functional oils on the modulation of mucosa-associated microbiota, intestinal health, and growth performance of nursery pigs. Animals 11, 1591.
Monda, H., Cozzolino, V., Vinci, G., Drosos, M., Savy, D., Piccolo, A., 2018. Molecular composition of the Humeome extracted from different green composts and their biostimulation on early growth of maize. Plant and Soil 429, 407-424.
Monda, H., Cozzolino, V., Vinci, G., Spaccini, R., Piccolo, A., 2017. Molecular characteristics of water-extractable organic matter from different composted biomasses and their effects on seed germination and early growth of maize. Science of the Total Environment 590, 40-49.
Moquet, P., Onrust, L., Van Immerseel, F., Ducatelle, R., Hendriks, W., Kwakkel, R., 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. World's Poultry Science Journal 72, 61-80.
Morrow, A.L., Lagomarcino, A.J., Schibler, K.R., Taft, D.H., Yu, Z., Wang, B., Altaye, M., Wagner, M., Gevers, D., Ward, D.V., 2013. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. Microbiome 1, 1-16.
Mowat, A.M., Agace, W.W., 2014. Regional specialization within the intestinal immune system. Nature Reviews Immunology 14, 667-685.

Mujnisa, A., Gustina, L., Natsir, A., Hasan, S., 2018. Research Article Dosage Effects of Lactococcus lactis ssp. lactis 2 as a Probiotic on the Percentage of Carcass, Abdominal Fat Content and Cholesterol Level in Broilers.
Mura, M., Andrade, C.F., Han, B., Seth, R., Zhang, Y., Bai, X.-H., Waddell, T.K., Hwang, D., Keshavjee, S., Liu, M., 2007. Intestinal ischemia-reperfusion-induced acute lung injury and oncotic cell death in multiple organs. Shock 28, 227-238.
Mutanen, A., Pakarinen, M.P., 2017. Serum fasting GLP-1 and GLP-2 associate with intestinal adaptation in pediatric onset intestinal failure. Clinical Nutrition 36, 1349-1354.
Namkung, H., Yu, H., Gong, J., Leeson, S., 2011. Antimicrobial activity of butyrate glycerides toward Salmonella Typhimurium and Clostridium perfringens. Poultry science 90, 2217-2222.
Navale, A.M., Paranjape, A.N., 2016. Glucose transporters: physiological and pathological roles. Biophysical reviews 8, 5-9.
Neath, C., Portocarero, N., Jones, C., 2022. In vitro susceptibility of swine pathogens to feed additives and active ingredients with potential as antibiotic replacements. Journal of Applied Microbiology 132, 1713-1723.
Negi, P.S., 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International journal of food microbiology 156, 7-17.
Nossol, C., Barta-Böszörményi, A., Kahlert, S., Zuschratter, W., Faber-Zuschratter, H., Reinhardt, N., Ponsuksili, S., Wimmers, K., Diesing, A.-K., Rothkötter, H.-J., 2015. Comparing two intestinal porcine epithelial cell lines (IPECs): morphological differentiation, function and metabolism. PloS one 10, e0132323.
Novik, G., Savich, V., 2020. Beneficial microbiota. Probiotics and pharmaceutical products in functional nutrition and medicine. Microbes and Infection 22, 8-18.
O'Hara, E., Kelly, A., McCabe, M.S., Kenny, D.A., Guan, L.L., Waters, S.M., 2018. Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. Scientific reports 8, 1-11.
Pan, L., An, D., Zhu, W., 2021. Low-tannin sorghum grain could be used as an alternative to corn in diet for nursery pigs. Journal of Animal Physiology and Animal Nutrition 105, 890-897.
Pancu, D.F., Scurtu, A., Macasoi, I.G., Marti, D., Mioc, M., Soica, C., Coricovac, D., Horhat, D., Poenaru, M., Dehelean, C., 2021. Antibiotics: Conventional Therapy and Natural Compounds with Antibacterial Activity—A Pharmaco-Toxicological Screening. Antibiotics 10, 401.
Papatsiros, V., Katsoulos, P.-D., Koutoulis, K., Karatzia, M., Dedousi, A., Christodoulopoulos, G., 2014. Alternatives to antibiotics for farm animals. CABI Reviews, 1-15.
Park, M., Cho, H., Jung, H., Lee, H., Hwang, K.T., 2014. Antioxidant and anti-inflammatory activities of tannin fraction of the extract from black raspberry seeds compared to grape seeds. Journal of food biochemistry 38, 259-270.
Parmar, A., Lakshminarayanan, R., Iyer, A., Mayandi, V., Leng Goh, E.T., Lloyd, D.G., Chalasani, M.L.S., Verma, N.K., Prior, S.H., Beuerman, R.W., 2018. Design and syntheses of highly potent teixobactin analogues against Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant enterococci (VRE) in vitro and in vivo. Journal of medicinal chemistry 61, 2009-2017.
Petri, D., Hill, J., Van Kessel, A., 2010. Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig. Livestock Science 133, 107-109.
Pieper, R., Tudela, C.V., Taciak, M., Bindelle, J., Pérez, J.F., Zentek, J., 2016. Health relevance of intestinal protein fermentation in young pigs. Animal health research reviews 17, 137-147.

Poder, T., Boileau, J.C., Lafrenière, R., Thibault, L., Carrier, N., de Grandmont, M.J., Beauregard, P., 2017. Quantitative assessment of haemolysis secondary to modern infusion pumps. Vox sanguinis 112, 201-209.
Prieto, A., López-Novo, C., Díaz, P., Díaz-Cao, J.M., López-Lorenzo, G., Antón, C., Remesar, S., García-Dios, D., López, C., Panadero, R., 2022. Antimicrobial Susceptibility of Enterotoxigenic Escherichia coli from Diarrhoeic Neonatal Calves in Spain. Animals 12, 264.
Priyadarshani, I., Rath, B., 2012. Commercial and industrial applications of micro algae–A review. Journal of Algal Biomass Utilization 3, 89-100.
Purohit, H.J., 2018. Gut-bioreactor and human health in future. Indian journal of microbiology 58, 3-7.
Pyo, J., Hare, K., Pletts, S., Inabu, Y., Haines, D., Sugino, T., Guan, L., Steele, M., 2020. Feeding colostrum or a 1: 1 colostrum: milk mixture for 3 days postnatal increases small intestinal development and minimally influences plasma glucagon-like peptide-2 and serum insulin-like growth factor-1 concentrations in Holstein bull calves. Journal of Dairy Science 103, 4236-4251.
Quan, G.-B., Han, Y., Yang, C., Hu, W.-B., Liu, M.-X., Liu, A., Wang, Y., Wang, J.-X., 2008. Inhibitory effect of trehalose on phosphatidylserine exposure, osmotic fragility and membrane lipid peroxidation damage of erythrocytes induced by high concentration of glucose. Zhongguo shi yan xue ye xue za zhi 16, 1442-1446.
Quosdorf, S., Schuetz, A., Kolodziej, H., 2017. Different inhibitory potencies of oseltamivir carboxylate, zanamivir, and several tannins on bacterial and viral neuraminidases as assessed in a cell-free fluorescence-based enzyme inhibition assay. Molecules 22, 1989.
Ran, T., Gomaa, W., Shen, Y., Saleem, A., Yang, W., McAllister, T., 2019. Use of naturally sourced feed additives (lactobacillus fermentation products and enzymes) in growing and finishing steers: Effects on performance, carcass characteristics and blood metabolites. Animal Feed Science and Technology 254, 114190.
Rasooly, R., Molnar, A., Choi, H.-Y., Do, P., Racicot, K., Apostolidis, E., 2019. In-vitro inhibition of staphylococcal pathogenesis by witch-hazel and green tea extracts. Antibiotics 8, 244.
Regulation EC 1831/2003, of the European Parliament and of the council, of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance).
Regulation EU 6/2019, Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC (Text with EEA relevance).
Ren, S., Chen, A., Tian, Y., Bai, Z., Wang, C., 2022. Lactobacillus paracasei from Koumiss Ameliorates Diarrhea in mice via Tight Junctions Modulation. Nutrition, 111584.
Rhayat, L., Jacquier, V., Brinch, K., Nielsen, P., Nelson, A., Geraert, P.-A., Devillard, E., 2017. Bacillus subtilis s train specificity affects performance improvement in broilers. Poultry Science 96, 2274-2280.
Rhouma, M., Fairbrother, J.M., Beaudry, F., Letellier, A., 2017. Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. Acta Veterinaria Scandinavica 59, 1-19.
Ricci, A., Olejar, K.J., Parpinello, G.P., Mattioli, A.U., Teslić, N., Kilmartin, P.A., Versari, A., 2016. Antioxidant activity of commercial food grade tannins exemplified in a wine model. Food Additives & Contaminants: Part A 33, 1761-1774.
Ricke, S., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry science 82, 632-639.
Rodrigues, A.M., Silva, D.B., Miranda, M.F., Braga da Silva, S.C., Canton Santos, L.E., Scorza, F.A., Scorza, C.A., Moret, M.A., Guimarães de Almeida, A.-C., 2021. The Effect of Low

Magnesium Concentration on Ictal Discharges In A Non-Synaptic Model. International Journal of Neural Systems 31, 2050070.
Rodrigues, F.F., Costa, J.G., Coutinho, H.D., 2009. Synergy effects of the antibiotics gentamicin and the essential oil of Croton zehntneri. Phytomedicine 16, 1052-1055.
Roselli, M., Pieper, R., Rogel-Gaillard, C., de Vries, H., Bailey, M., Smidt, H., Lauridsen, C., 2017. Immunomodulating effects of probiotics for microbiota modulation, gut health and disease resistance in pigs. Animal Feed Science and Technology 233, 104-119.
Ross, A.C., Caballero, B., Cousins, R.J., Tucker, K.L., Ziegler, T.R., 2012. Modern nutrition in health and disease. Lippincott Williams & Wilkins.
Rossi, L., Dell'Orto, V., Vagni, S., Sala, V., Reggi, S., Baldi, A., 2014. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic Escherichia coli strain in piglets. Veterinary Research Communications 38, 39-49.
Rossi, L., Di Giancamillo, A., Reggi, S., Domeneghini, C., Baldi, A., Sala, V., Dell'Orto, V., Coddens, A., Cox, E., Fogher, C., 2013. Expression of verocytotoxic Escherichia coli antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. Journal of Veterinary Science 14, 263-270.
Rossi, L., Turin, L., Alborali, G.L., Demartini, E., Filipe, J.F.S., Riva, F., Riccaboni, P., Scanziani, E., Trevisi, P., Dall'Ara, P., 2021. Translational Approach to Induce and Evaluate Verocytotoxic E. coli O138 Based Disease in Piglets. Animals 11, 2415.
Roto, S.M., Rubinelli, P.M., Ricke, S.C., 2015. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. Frontiers in Veterinary Science 2, 28.
Sahin, K., Orhan, C., Tuzcu, M., Ali, S., Sahin, N., Hayirli, A., 2010. Epigallocatechin-3-gallate prevents lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors in heat-stressed quails. Poultry Science 89, 2251-2258.
Salehi, B., Martorell, M., Arbiser, J.L., Sureda, A., Martins, N., Maurya, P.K., Sharifi-Rad, M., Kumar, P., Sharifi-Rad, J., 2018. Antioxidants: positive or negative actors? Biomolecules 8, 124.
Salehi, P., Zhu, J.Z., Castillo, E.G., Avila, J., Lakey, J., Churchill, T.A., 2003. Preserving the mucosal barrier during small bowel storage1. Transplantation 76, 911-917.
Samat, N.A., Yusoff, F.M., Rasdi, N.W., Karim, M., 2020. Enhancement of live food nutritional status with essential nutrients for improving aquatic animal health: A review. Animals 10, 2457.
Samudovska, A., Demeterova, M., 2010. Effect of diet supplemented with natural humic compounds and sodium humate on performance and selected metabolic variables in broiler chickens. Acta Veterinaria Brno 79, 385-393.
Saw, C.L.L., Yang, A.Y., Cheng, D.C., Boyanapalli, S.S.-S., Su, Z.-Y., Khor, T.O., Gao, S., Wang, J., Jiang, Z.-H., Kong, A.-N.T., 2012. Pharmacodynamics of ginsenosides: antioxidant activities, activation of Nrf2, and potential synergistic effects of combinations. Chemical Research in Toxicology 25, 1574-1580.
Schokker, D., Fledderus, J., Jansen, R., Vastenhouw, S.A., de Bree, F.M., Smits, M.A., Jansman, A.A., 2018. Supplementation of fructooligosaccharides to suckling piglets affects intestinal microbiota colonization and immune development. Journal of animal science 96, 2139-2153.
Scicutella, F., Mannelli, F., Daghio, M., Viti, C., Buccioni, A., 2021. Polyphenols and organic acids as alternatives to antimicrobials in poultry rearing: a review. Antibiotics 10, 1010.
Scillitani, A., Pugliese, F., Salcuni, A.S., Carnevale, V., 2020. Regolazione del metabolismo del calcio, del fosfato e del magnesio. L'Endocrinologo 21, 348-353.

Selim, S., Alfy, S., Al-Ruwaili, M., Abdo, A., Jaouni, S., 2012. Susceptibility of imipenem-resistant Pseudomonas aeruginosa to flavonoid glycosides of date palm (Phoenix dactylifera L.) tamar growing in Al Madinah, Saudi Arabia. African Journal of biotechnology 11, 416-422.
Serrano, J., Puupponen-Pimiä, R., Dauer, A., Aura, A.M., Saura-Calixto, F., 2009. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. Molecular nutrition & food research 53, S310-S329.
Settle, T., Leonard, S., Falkenstein, E., Fix, N., Van Dyke, K., Klandorf, H., 2014. Effects of a phytogenic feed additive versus an antibiotic feed additive on oxidative stress in broiler chicks and a possible mechanism determined by electron spin resonance. International journal of poultry science 13, 62.
Shah, J., 2003. The salicylic acid loop in plant defense. Current opinion in plant biology 6, 365-371.
Shahidi, F., Zhong, Y., 2015. Measurement of antioxidant activity. Journal of functional foods 18, 757-781.
Shanahan, F., van Sinderen, D., O'Toole, P.W., Stanton, C., 2017. Feeding the microbiota: transducer of nutrient signals for the host. Gut 66, 1709-1717.
Shin, N.-R., Whon, T.W., Bae, J.-W., 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology 33, 496-503.
Sindhi, V., Gupta, V., Sharma, K., Bhatnagar, S., Kumari, R., Dhaka, N., 2013. Potential applications of antioxidants–A review. Journal of pharmacy research 7, 828-835.
Sjöberg, Å., Lutz, M., Tannergren, C., Wingolf, C., Borde, A., Ungell, A.-L., 2013. Comprehensive study on regional human intestinal permeability and prediction of fraction absorbed of drugs using the Ussing chamber technique. European Journal of Pharmaceutical Sciences 48, 166-180.
Smith, G., 2015. Antimicrobial decision making for enteric diseases of cattle. Veterinary Clinics: Food Animal Practice 31, 47-60.
Smith, G.W., Berchtold, J., 2014. Fluid therapy in calves. Veterinary Clinics: Food Animal Practice 30, 409-427.
Sotira, S., Dell'Anno, M., Caprarulo, V., Hejna, M., Pirrone, F., Callegari, M.L., Tucci, T.V., Rossi, L., 2020. Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood Metabolites and Gut Microbiota in Weaned Piglets. Animals 10, 726.
Sreenivasan, P.K., Kakarla, V.V.P., Sharda, S., Setty, Y., 2021. The effects of a novel herbal toothpaste on salivary lactate dehydrogenase as a measure of cellular integrity. Clinical oral investigations 25, 3021-3030.
Stangl, M., Krapp, J., Theodorou, D., Eder, M., Hammer, C., Land, W., Schildberg, F.W., 2000. Computer-assisted ex vivo, normothermic small bowel perfusion. European surgical research 32, 100-106.
Stefańska, B., Sroka, J., Katzer, F., Goliński, P., Nowak, W., 2021. The effect of probiotics, phytobiotics and their combination as feed additives in the diet of dairy calves on performance, rumen fermentation and blood metabolites during the preweaning period. Animal Feed Science and Technology 272, 114738.
Sukumar, M.R., König, B., 2018. Pomegranate extract specifically inhibits Clostridium difficile growth and toxin production without disturbing the beneficial bacteria in vitro. Infection and Drug Resistance 11, 2357.
Sullivan, I., Faulds, J., 2013. Lactate dehydrogenase and Haemolysis Index as quality control markers of haemolysis in intra-operative cell salvage. Transfusion medicine 23, 326-329.

Sundaram, T.S., Giromini, C., Rebucci, R., Baldi, A., 2020. Omega-3 polyunsaturated fatty acids counteract inflammatory and oxidative damage of non-transformed porcine enterocytes. Animals 10, 956.
Suresh, G., Das, R.K., Kaur Brar, S., Rouissi, T., Avalos Ramirez, A., Chorfi, Y., Godbout, S., 2018. Alternatives to antibiotics in poultry feed: molecular perspectives. Critical reviews in microbiology 44, 318-335.
Sutton, M.E., op den Dries, S., Karimian, N., Weeder, P.D., de Boer, M.T., Wiersema-Buist, J., Gouw, A.S., Leuvenink, H.G., Lisman, T., Porte, R.J., 2014. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PloS one 9, e110642.
Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L., 2014. The role of short-chain fatty acids in health and disease. Advances in immunology 121, 91-119.
Tang, Y., 2022. Nutraceuticals, p. 102265.
Terré, M., Ortuzar, I., Graffelman, J., Bassols, A., Vidal, M., Bach, A., 2021. Using compositional mixed-effects models to evaluate responses to amino acid supplementation in milk replacers for calves. Journal of dairy science 104, 7808-7819.
Thuong, M., Ruiz, A., Evrard, P., Kuiper, M., Boffa, C., Akhtar, M.Z., Neuberger, J., Ploeg, R., 2016. New classification of donation after circulatory death donors definitions and terminology. Transplant International 29, 749-759.
Tiedje, J.M., 2019. Environmental aspects of antibiotic resistance, Oxford University Press.
Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2017. Effects of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on the health, oxidative stress status and fatty acid profile in weaned piglets. Veterinarni Medicina 62, 16-28.
Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2018. The effect of leonardite and lignite on the health of weaned piglets. Research in Veterinary Science 119, 134-142.
Uc, A., Olivier, A.K., Griffin, M.A., Meyerholz, D.K., Yao, J., Abu-El-Haija, M., Buchanan, K.M., Vanegas Calderón, O.G., Abu-El-Haija, M., Pezzulo, A.A., 2015. Glycaemic regulation and insulin secretion are abnormal in cystic fibrosis pigs despite sparing of islet cell mass. Clinical science 128, 131-142.
Udensi, U.K., Tchounwou, P.B., 2017. Potassium homeostasis, oxidative stress, and human disease. International journal of clinical and experimental physiology 4, 111.
Upadhyay, A., Johny, A.K., Amalaradjou, M.A.R., Baskaran, S.A., Kim, K.S., Venkitanarayanan, K., 2012. Plant-derived antimicrobials reduce Listeria monocytogenes virulence factors in vitro, and down-regulate expression of virulence genes. International Journal of Food Microbiology 157, 88-94.
Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K., 2014. Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. BioMed research international 2014.
Valenzuela-Grijalva, N.V., Pinelli-Saavedra, A., Muhlia-Almazan, A., Domínguez-Díaz, D., González-Ríos, H., 2017. Dietary inclusion effects of phytochemicals as growth promoters in animal production. Journal of animal science and technology 59, 1-17.
Vase-Khavari, K., Mortezavi, S.-H., Rasouli, B., Khusro, A., Salem, A.Z., Seidavi, A., 2019. The effect of three tropical medicinal plants and superzist probiotic on growth performance, carcass characteristics, blood constitutes, immune response, and gut microflora of broiler. Tropical animal health and production 51, 33-42.

Veklich, T., IuIu, M., Kosterin, S., 2015. Mg2+, ATP-dependent plasma membrane calcium pump of smooth muscle cells. I. Structural organization and properties. Ukrainian biochemical journal 87, 5-20.
Velasco, V., Williams, P., 2011. Improving meat quality through natural antioxidants. Chilean journal of agricultural research 71, 313.
Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, H., 2015. The impact of food bio-actives on gut health. Vitro and ex vivo models, 83-93.
Verrillo, M., Salzano, M., Savy, D., Di Meo, V., Valentini, M., Cozzolino, V., Piccolo, A., 2022. Antibacterial and antioxidant properties of humic substances from composted agricultural biomasses. Chemical and Biological Technologies in Agriculture 9, 1-15.
Vormann, J., 2003. Magnesium: nutrition and metabolism. Molecular aspects of medicine 24, 27-37.
Waguespack, A., Powell, S., Roux, M., Fruge, E., Bidner, T., Payne, R., Southern, L., 2011. Effect of determining baseline plasma urea nitrogen concentrations on subsequent posttreatment plasma urea nitrogen concentrations in 20-to 50-kilogram pigs. Journal of animal science 89, 4116-4119.
Walencka, E., Rozalska, S., Wysokinska, H., Rozalski, M., Kuzma, L., Rozalska, B., 2007. Salvipisone and aethiopinone from Salvia sclarea hairy roots modulate staphylococcal antibiotic resistance and express anti-biofilm activity. Planta medica 73, 545-551.
Wall, E.H., Doane, P.H., Donkin, S.S., Bravo, D., 2014. The effects of supplementation with a blend of cinnamaldehyde and eugenol on feed intake and milk production of dairy cows. Journal of Dairy science 97, 5709-5717.
Wang, M., Wang, R., Liu, M., Beauchemin, K., Sun, X., Tang, S., Jiao, J., Tan, Z., He, Z., 2019a. Dietary starch and rhubarb supplement increase ruminal dissolved hydrogen without altering rumen fermentation and methane emissions in goats. animal 13, 975-982.
Wang, N., Wu, W., Pan, J., Long, M., 2019b. Detoxification strategies for zearalenone using microorganisms: A review. Microorganisms 7, 208.
Wang, Q., Chen, Y., Yoo, J., Kim, H., Cho, J., Kim, I., 2008. Effects of supplemental humic substances on growth performance, blood characteristics and meat quality in finishing pigs. Livestock Science 117, 270-274.
Wang, Q., Kim, H., Cho, J., Chen, Y., Yoo, J., Kim, I., 2007. Effects of supplemental humic substances on egg production and quality in laying hens, JOURNAL OF DAIRY SCIENCE, AMER DAIRY SCIENCE ASSOC 1111 N DUNLAP AVE, SAVOY, IL 61874 USA, pp. 519-519.
Wealleans, A., Sirukhi, M., Egorov, I., 2017. Performance, gut morphology and microbiology effects of a Bacillus probiotic, avilamycin and their combination in mixed grain broiler diets. British poultry science 58, 523-529.
WHO, 2019. World Health Organization. Critically important antimicrobials for human medicine.
WHO, 2022. World Health Organization. Antimicrobial resistance surveillance in Europe 2022–2020 data.
Whon, T.W., Kim, H.S., Shin, N.-R., Sung, H., Kim, M.-S., Kim, J.Y., Kang, W., Kim, P.S., Hyun, D.-W., Seong, H.J., 2021. Calf diarrhea caused by prolonged expansion of autochthonous gut Enterobacteriaceae and their lytic bacteriophages. Msystems 6, e00816-00820.
Windeyer, M., Leslie, K., Godden, S.M., Hodgins, D., Lissemore, K., LeBlanc, S., 2014. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Preventive veterinary medicine 113, 231-240.

Yadav, S., Jha, R., 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. Journal of animal science and biotechnology 10, 1-11.
Yang, I., Corwin, E.J., Brennan, P.A., Jordan, S., Murphy, J.R., Dunlop, A., 2016. The infant microbiome: implications for infant health and neurocognitive development. Nursing research 65, 76.
Yang, W., Yu, T., Huang, X., Bilotta, A.J., Xu, L., Lu, Y., Sun, J., Pan, F., Zhou, J., Zhang, W., 2020. Intestinal microbiota-derived short-chain fatty acids regulation of immune cell IL-22 production and gut immunity. Nature communications 11, 1-18.
Yeo, S., Lee, S., Park, H., Shin, H., Holzapfel, W., Huh, C.S., 2016. Development of putative probiotics as feed additives: validation in a porcine-specific gastrointestinal tract model. Applied microbiology and biotechnology 100, 10043-10054.
Yim, G., McClure, J., Surette, M.G., Davies, J.E., 2011. Modulation of Salmonella gene expression by subinhibitory concentrations of quinolones. The Journal of antibiotics 64, 73-78.
Yudiarti, T., Isroli, I., Yunianto, V., 2020. Nutritive and antioxidative properties of some selected agro-industrial by-products fermented with the fungus Chrysonillia crassa as alternative feedstuffs for poultry, Journal of Physics: Conference Series, IOP Publishing, p. 012145.
Zhang, L., Wu, W., Lee, Y.-K., Xie, J., Zhang, H., 2018. Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. Frontiers in microbiology 9, 48.
Zhang, R., Yang, S., An, Y., Wang, Y., Lei, Y., Song, L., 2022a. Antibiotics and antibiotic resistance genes in landfills: A review. Science of The Total Environment 806, 150647.
Zhang, W.-X., Zhang, Y., Zhang, X.-W., Deng, Z.-X., Liu, J.-X., He, M.-L., Wang, H.-F., 2020. Effects of Dietary Supplementation with Combination of Tributyrin and Essential Oil on Gut Health and Microbiota of Weaned Piglets. Animals 10, 180.
Zhang, Z., Zhang, G., Zhang, S., Zhao, J., 2022b. Fructooligosaccharide Reduces Weanling Pig Diarrhea in Conjunction with Improving Intestinal Antioxidase Activity and Tight Junction Protein Expression. Nutrients 14, 512.
Zhou, Z., Packialakshmi, B., Makkar, S., Dridi, S., Rath, N., 2014. Effect of butyrate on immune response of a chicken macrophage cell line. Veterinary Immunology and Immunopathology 162, 24-32.

7 General Discussion and Conclusions

WHO estimated that world population is constantly growing and by 2050 global population will increase to 9.8 billion. To fulfil the increasing food demand, we have to face important challenges related to sustainability and health. In particular, antimicrobial resistance is a global worldwide threat for humans, animals and the environment. Spreading of antimicrobial resistance genes will potentially lead to a progressive ineffectiveness of antibiotic treatments, causing the lack of medical cares for bacterial infections. This problem prompted countries from around the world to establish restrictive regulations for a judicious use of antibiotics in order to preserve the last effective molecules. On the other hand, research is aiming to find possible alternatives or integrated approaches to contrast this emerging issue.

Livestock farming require to evolve due to the current needing of a more efficient and sustainable production system. During their first phases of life, animals have to face several stressors that can increase the risk of development of various multifactorial diseases which comprise gastrointestinal disorders. These diseases have a negative reflect on farm profitability and animal health due to the cost of treatment and decreased growth performance. The presence of pathologies resort farmers to use antibiotics treatments to heal affected animals. The nutritional approach can be considered interesting for its functionality by supplementing diets with bioactive compounds capable of increasing health status of animals and preventing pathologies occurrence. Functional feed additives can be considered as interesting alternative due to their content of nutricines that have a positive impact on animal health.

In this thesis algae, tannins extract, tributyrin, leonardite, *L. plantarum* and *L. reuteri* have been evaluated with *in vitro* and/or *in vivo* approaches as alternatives to antibiotics.

The *in vitro* evaluation allowed to assess the main functional properties of investigated additives, permitting to optimize their inclusion rate and reducing the use of animals for experimental purposes in line with the 3R principles.

The characterization of algae showed that they might be promising for their particular functionality with antimicrobial and antioxidant activities. In addition, algae species are unique presenting different characteristics, and they may be combined to exploit their complementary effects in order to create synergistic combinations.

Tannins have been shown to be the strongest antioxidant and antimicrobial additives among tested compounds showing different ability depending on their source (quebracho or chestnut). Tannins can create insoluble bonds with dietary proteins leading to lower bioaccessibility and for this reason their supplementation in feed

should be optimized to maximise their functional effects without impairing protein digestibility.

L. plantarum and *L. reuteri* are not included in the Register of Feed Additives of EU (Reg. 1831/2003) as functional additives for swine. However, our results showed that they demonstrated to resist to gastric pH, to simulated gastrointestinal process, to have co-aggregative properties and to possess adhesive capacity on swine enterocytes mainly due to their surface proteins. These characteristics suggested the possible use of *L. plantarum* and *L. reuteri* as interesting probiotics for swine species.

The *in vivo* evaluation exacerbated some features of functional feed additives in field conditions. In particular, the supplementation of 1.25% of tannins extract did not affect feed palatability and did not influence the performance. However, the polyphenolic intake and the nitrogen excretion was higher in the treatment group. Tannins supplementation lowered serum urea concentration after 42 days. Results on intestinal cells *in vitro* revealed a hermetic effect of tannins and their capacity to counteract oxidative stress enhancing the cell viability. These results suggested that tannins have potent antioxidant, antimicrobial, and citomodulatory effect, however their positive impact can be better elucidated under stressful conditions.

The dietary inclusion of 0.20% of tributyrin positively increased zootechnical performance, lowered urea concentration and modulated cholesterol concentration. Tributyrin supplementation decreased the relative abundance of *Bifidobacteria* spp. and *Lactobacillus* spp. that are recognised as positive indicators of gut health.

Leonardite supplemented in feed at 0.25% significantly raised zootechnical performance of piglets, modulated lipid metabolism and increased mineral serum content. In addition, leonardite showed antioxidant activity due to their phenolic moieties and carboxylic acids without highlighting differences in serum diamine oxidase concentration thus suggesting that DAO variations are related to the presence of intestinal damage. Leonardite supplementation lowered the abundance of *Bifidobacteria* spp. and *Lactobacillus* spp. and an increase amount of *Prevotella* genera.

These results seem to indicate that functional feed additives may decrease some positive indicators of gut health. The needing to investigate this aspect to better understand mechanisms that regulates the microbial environment of gut and their possible modulation by dietary approach was explored by the evaluation of gut microbiota by V3-V6 region of 16S rRNA gene amplification and sequencing.

Microbiota evaluation of tannins supplemented in piglets revealed to favour groups of butyrate-producing bacteria and microbial pathways with a higher potential for carbohydrate metabolism. Tributyrin supplementation showed the highest impact on gut microbiota increasing the beta-diversity of treated group compared to control. In particular, tributyrin supplementation increase the relative abundance of *Oscillospira*,

Oscillibacter, *Mucispirillum* and *Butyrivibrio* genera positively correlated to animal growth. Using the functional profile prediction, tributyrin group revealed a higher genetic potential for energy metabolism and a lowered potential for carbohydrate metabolism. Depth investigation of gut microbiota was useful for enriching the scientific knowledge related to gut microbiota modulation of functional feed additives. Due to encouraging results observed during *in vivo* trials with tannins, tributyrin and leonardite supplementation, a possible combined effect of these three functional compounds was investigated in weaned piglets with a combined supplementation for 28 days. Results showed that mixture of tannins extract, leonardite and tributyrin significantly decreased diarrhoea occurrence, modulated the lipid metabolism, increased the abundance of *Prevotella* and *Fibrobacter* genera and lactic acid:coliform bacteria ratio. Based on these promising results the use of different functional compounds characterized by different mechanisms of action may positively contribute to intestinal health and diarrhoea prevention in piglets thus decreasing antibiotic treatments.

L. plantarum and *L. reuteri* supplemented in weaned piglets significantly decreased the diarrhoea incidence in treated groups compared to control showing a higher faecal consistency. In particular *L. plantarum* registered the lowest cases of diarrhoea over 28 days of trial compared to other groups. The dietary supplementation of the combination of both bacterial strains did not show a particular advantage on animal health and performance suggesting that they do not exert a complementary or synergistic effect.

Due to increasing public awareness and concern on animal experimentation, it is necessary to develop innovative science-based model that can predict nutrient value and their bioaccessibility without using animals for experimental purposes. The use of the most innovative *ex vivo* perfusion techniques allow to study and mimic the intestinal absorption obtaining reliable data for translation to *in vivo*. For this reason, an intestinal swine perfused segment model was developed to explore possible alternatives to animal experimentation. Obtained findings confirmed nutrient bioaccessibility maintaining most of histological structure of the organ. This encouraging result can be used for improving *ex vivo* perfused models for future investigations on nutrients bioaccessibility and translational medicine.

In conclusion, tested compounds can not substitute the therapeutic treatment of antibiotics. However, the utilization dietary approaches using functional feed additive can significantly contribute to decreasing pathology occurrence, boosting animal health and performance thus decreasing the needing of therapeutic treatments leading to a reduction in antibiotics use. In addition, possible synergistic effects have to be

considered by combining various compounds with different mechanisms of action for improving the health status, intestinal eubiosis and animal resiliency.
The following project contributed to increase the knowledge related to bioactive compounds capable to boost the animal health, prevent pathologies occurrence and raise farm profitability. Nowadays, costs related to dietary inclusion of feed additives are high and their use could be more convenient for farms where high prevalence of pathogens and gastrointestinal disorders has been registered. Some of these functional products, such as algae, could probably find their application with innovative cultivation systems directly in the farm. Other compounds can be obtained from the valorization of agro-industrial wastes such as tannins which may lead to lower costs of functional additives on the market.
In line with the One Health principles, the reduction of antibiotics use will be fundamental for preserving the last effective antibiotic molecules and safeguarding the environment for a future sustainable development on Earth. In light of this, the use of functional feed additives can be considered as interesting alternative for preventing multifactorial disorders and thus reducing the antibiotic treatments in livestock farming.

7.1 Future Goals and Perspectives:

This long journey through my PhD allowed me to importantly increase my scientific knowledge on alternatives to antimicrobial related to feed additives. However, many questions and points need to be addressed by further studies. Based on obtained findings, I strongly believe that dietary supplementation of functional feed additives could contribute to the decrease of antimicrobial in livestock thus reducing resistance genes spreading.
Today, another fundamental key point in line with the One Health principles relies on the animal farming sustainability. The livestock system is currently debated for various prospectives related to the environment in regard to the water and land use, pollution, deforestation and the competition with humans for the cultivation of crops for food production. In line with new international policies for the future world development, strategies to mitigate the environmental pollution and reduce the impact of animal farming are required. In this scenario, the exploitation of agro-chain wastes for animal nutrition could represent an interesting strategy in order to enhance the circular economy of farms reducing the environmental impact. The dietary approach represents one of the most effective strategies for modulating nutrients excretion, production of wastes and greenhouse gasses emission. The aim of future projects will be to develop innovative dietary strategies and feed additives that

favourably affect the environment or stabilise the physiological conditions in order to increase animal health, performance, thus reducing the antibiotic use and environmental impact of livestock productions. Specific aims future work will be focused on i) the reduction of antibiotic use in animal farming, ii) the increase of livestock sustainability valorizing wastes and reducing emissions. The idea will be to investigate natural substances, bioactive effects from plant-based products and edible vaccines as possible alternatives to antibiotics and environmentally sustainable products through *in vitro*, *ex vivo* and *in vivo* approaches, following the 3R principle on animal experimentation (Dir. 2010/63/EU). Future projects wish to evaluate innovative functional feed ingredients, additives, bioactive compounds and engineered edible vaccines through multiple approaches assessing their composition, digestibility, antioxidant capacity, antimicrobial and immunogenic effects and their impact on the environment, animal health and performance.

In particular two interesting topics could be considered according to current aims of European Union and international organizations: i) the valorization of plant wastes from the agro-chain production as feed additives alternative to antibiotics (e.g. chestnut waste and hemp hurds) and ii) the development of innovative edible vaccines against enteric diseases caused by pathogenic Escherichia coli of swine species. Edible vaccines study could continue an initiated project developed during my PhD carrier parallelly with the research group and during my internship at the Laboratory of Immunology at the University of Ghent under the supervision of Prof. Eric Cox. Oral vaccines could become an important sustainable alternative to the traditional antigen delivery for their cost-effective, needleless, convenient, safe alternative to vaccine production. In particular, the attention will be focalized on the evaluation of xenogenic tobacco seeds and engineered *Lactococcus lactis* strains as multivalent edible vaccinates against major *E. coli* pathotypes for swine.