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Optimizing monogastrics and ruminants gut health to face weaning transition challenges

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Abstract

The weaning transition represents one of the most critical phases of the cycle of production, in which monogastrics and ruminants are susceptible to environmental, social, and nutritional stressors that may favor the outburst of multifactorial diseases. Among them, gastroenteric pathologies represent a major cause of economic loss and antimicrobials usage. Feed additives represent a key tool to shape the gut health of monogastrics and ruminants during weaning transition. As depicted by current European legislation, antimicrobials and pharmacological dosages of zinc and copper for growth-promoting purposes are no longer allowed due to public health and environmental issues which brought lower limits of inclusion in feeds, especially concerning zinc and copper. Continuous research of different solutions able to guarantee an effective modulation of gut health is strongly needed. Weaning changes in the gut environment involves a dynamic interplay between the gut barrier and the microbiota. Nevertheless, the balance among microbial niches and the enteric environment can be perturbed during weaning. The purpose of the present thesis was to evaluate different nutritional strategies based on feed additives and verify their effects on gut health and microbiota of piglets and dairy calves during weaning. In **chapter 6** is described an *in vitro* characterization of a blend of carvacrol, tannic acid and medium chain fatty acids (MCFAs) supplemented in weanling piglets. Published results showed the potential of the blend in ameliorating gut barrier integrity and morphological characteristics, having notable reflexes in terms of salivary cortisol as biomarker of intestinal health, fecal score and gut microbiota composition. After the ban on pharmacological dosages of zinc and copper, the need to deeply investigate their relationship in dietary interventions was particularly evident. In **chapter 7**, the administration of different ratios of zinc and copper administered via specialty oxide sources in weanling piglets was evaluated. The unbalanced administration of Zn and Cu can be detrimental to intestinal permeability and immune parameters during early post-weaning phases, as confirmed also by evident microbiota variations in terms of compositional distances and taxa. The dietary supplementation of *Bacillus spp*. strains has not been extensively investigated in calves during weaning. A third study (**chapter 8**) was focused on the administration of *Weizmannia faecalis* strain DSM32016 in Italian female Holstein calves' diet. Results highlighted the effectiveness of the treatment in conditioning the circulating immunoglobulins level, the antioxidant status and the fecal microbiota features, ameliorating growth performance and fecal score. The possibility to evaluate further alternative strategies to counterattack diarrheal disorders in piglets was explored during a 6-month secondment at the Department of Animal and Veterinary Sciences of Aarhus University (Denmark). In particular, an immunostimulant parenteral vaccine targeting secretory IgA production was tested to prevent *E. coli* induced post weaning diarrhea. As this project is on-going, a brief report of this experience is reported in **chapter 9.** As discussed in **chapter 10**, the tested solutions outlined their validity as a preventive tool to enhance gut health and animals' performance during weaning also by establishing a balanced microbiota, possibly promoting the reduction of antimicrobial-based treatments and decreasing the manifestation of gastroenteric disorders.

La transizione allo svezzamento rappresenta una delle fasi più critiche del ciclo produttivo, in cui monogastrici e ruminanti sono suscettibili a fattori di stress ambientali, sociali e nutrizionali che possono favorire l'insorgenza di patologie multifattoriali. Tra queste, le patologie gastroenteriche rappresentano una delle principali cause di perdita economica e di utilizzo di antimicrobici. Gli additivi per mangimi rappresentano uno strumento chiave per modulare la salute enterica di monogastrici e ruminanti durante la transizione allo svezzamento. Come previsto dall'attuale legislazione europea, gli antimicrobici e i dosaggi farmacologici di zinco e rame come promotori di crescita non sono più consentiti a causa di problemi legati alla salute pubblica e all'inquinamento ambientale, che hanno portato a limiti più bassi di inclusione nei mangimi, in particolare per quanto riguarda zinco e rame. la continua ricerca di soluzioni diverse in grado di garantire un'efficace modulazione della salute dell'intestino è fortemente necessaria. I cambiamenti dello svezzamento nell'ambiente intestinale comportano un'interazione dinamica tra la barriera intestinale e il microbiota. Tuttavia, l'equilibrio tra le nicchie microbiche e l'ambiente enterico può essere perturbato durante lo svezzamento. Lo scopo della presente tesi è stato quello di valutare diverse strategie nutrizionali basate su additivi per mangimi e verificare i loro effetti sulla salute dell'intestino e sul microbiota di suinetti e vitelli da latte durante la transizione allo svezzamento. Nel **capitolo 6** viene descritta una caratterizzazione *in vitro* di una miscela di carvacrolo, acido tannico e acidi grassi a catena media (MCFA) integrata nella dieta di suinetti svezzati. I risultati pubblicati hanno mostrato il potenziale della miscela nel migliorare l'integrità della barriera intestinale e le relative caratteristiche morfologiche, avendo notevoli riflessi sul cortisolo salivare come biomarcatore della salute intestinale, sul fecal score e sulla composizione del microbiota intestinale. Dopo il bando dei dosaggi farmacologici di zinco e rame, è emersa in modo particolarmente evidente la necessità di indagare a fondo la loro relazione biologica durante gli interventi nutrizionali. Nel **capitolo 7**, è stata valutata la somministrazione di diversi rapporti di zinco e rame somministrati tramite fonti maggiormente biodisponibili nelle diete per suinetti in svezzamento. La somministrazione sbilanciata di Zn e Cu può essere dannosa per la permeabilità intestinale e i parametri immunitari durante le prime fasi post-svezzamento, come confermato anche da evidenti variazioni del microbiota in termini di distanze composizionali e taxa. L'integrazione alimentare dei ceppi di *Bacillus* spp. non è stata ampiamente studiata nei vitelli durante lo svezzamento. Un terzo studio (**capitolo 8**) era focalizzato sulla somministrazione del ceppo *Weizmannia faecalis* DSM32016 in diete destinate a vitelle Holstein. I risultati hanno evidenziato l'efficacia del trattamento nel condizionare il livello di immunoglobuline circolanti, lo stato antiossidante e le caratteristiche del microbiota fecale, migliorando le prestazioni di crescita e il fecal score. La possibilità di valutare ulteriori strategie alternative per contrastare i disturbi diarroici nei suinetti è stata esplorata durante uno stage di 6 mesi presso il dipartimento di Scienze Animali e Medicina Veterinaria dell'Università di Aarhus (Danimarca). In particolare, è stato testato un vaccino parenterale immunostimolante mirato alla produzione di IgA secretorie per prevenire la diarrea post-svezzamento indotta da E. coli. Poiché questo progetto è attualmente in corso, un breve resoconto di questa esperienza è riportato nel **capitolo 9**. Come discusso nel **capitolo 10**, le soluzioni testate hanno delineato la loro validità come strumento preventivo utile a migliorare la salute intestinale e le prestazioni produttive degli animali durante lo svezzamento, favorendo un normale sviluppo del microbiota intestinale, e promuovendo la diminuzione dei disturbi gastroenterici.

CHAPTER 1: Introduction

1.1. General Scenario

The growing necessity to increase the sustainability of the livestock sector represents a direct consequence of the increase in demographic prospects. In particular, the World Health Organization (WHO) estimated that by 2050 the world population will rise until almost reaching 10 billion people. Contextually, animal by product production has been strongly influenced by the recent socio-economical impacting events. The outburst of Covid-19 caused a decrease in meat production from 338.9 million tons (carcass weight) in 2019 to 333.0 million tons in 2020. During the frame period 2018-2020, the decrease of pork meat production worldwide was attested at 19.9 million tons (Ijaz et al, 2021). More recently, the effects of the propagation of African Swine Fever (ASF) in China and Europe has been evident. Specifically, ASF outbreak caused the enhancement of substitute meat prices on the market, because of pork retail sector increased prices and economically damaged supply chain side in eastern countries (Acosta et al, 2023). In Europe, a similar situation is already happening in countries such as Romania, in which since the first outbreak in 2017 a cumulative value of € 1.245 billion was lost due to ASF (Ladoşi et al, 2023). In Italy, wild boars profuse proliferation and distribution contributed in September 2023 to four new incursions of ASF genotype II in the peninsular zone, following the eradication of genotype I in Sardinia. To date, restriction zones are routinely amended on the territory, and it is evident how these interventions can economically affect the pork trade market, especially considering the export side (Rosamilla et al, 2024; Pavone et al, 2023, 2024).

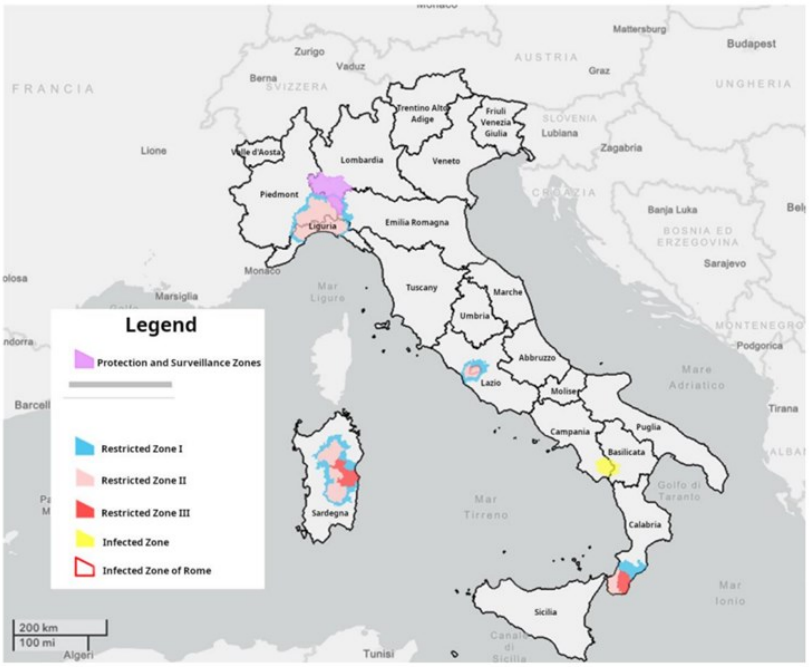

Figure 1. Epidemiological scenario in Italy (11th September 2023) with restriction zones (I, II and III) in compliance with the Commission Implementing Regulation (EU) 2023/1485 on the 18th of July 2023 (Pavone et al., 2023).

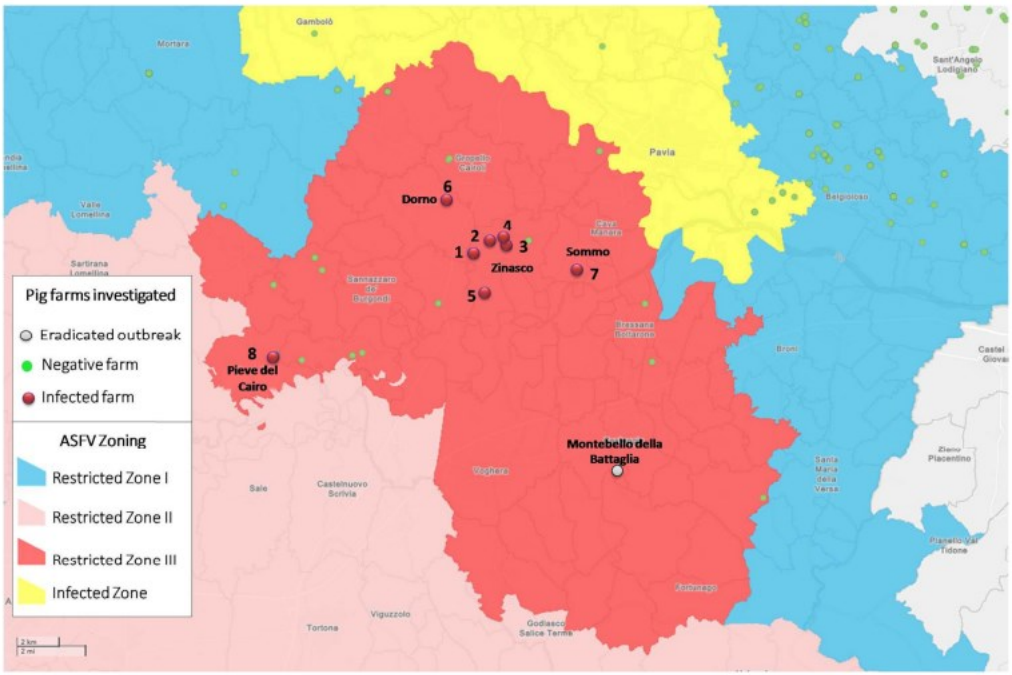

Figure 2. Detailed epidemiological situation and updated restriction zones distribution in north Italy updated to April 2024. In picture, Pavia's municipalities in which the ASF outbreaks were registered are numerated in chronological order (Pavone et al., 2024).

From a socio-economical point of view, the recent tensions among Russia and Ukraine had worldwide reflexes in terms of costs of production and transport of raw materials. The feed sector has been deeply affected in this sense. Russia and Ukraine were ranked top ten producers for maize, wheat and soybean. Nevertheless, war caused a decisive change in Ukraine production and export capacities, reflecting the increase of crop prices (i.e., wheat and barley) in several European countries for which Ukraine was considered a primary source (Lang T and McKee, 2022). Furthermore, in different countries raw material such as soybean meal and vegetal oils prices markedly increased. Moreover, it has to be considered that war started during sowing season for spring crop (March 2022). Thus, limitations in terms of sowing and exporting due to war for pivotal cultivars favored an additional cost raise for feed production and commercialization (Ben Hassen and El Bilali, 2022). Consequently, prices of various feed ingredients, which were already raised during Covid-19 outbreak, reached their highest (Nasir et al., 2022).

Period	**Wheat**	**Soybean**	**Maize**
January 2017-December 2019	200.05 ± 15.08	384.27 ± 21.99	163.44 ± 10.85
January 2020- February 2022 (Covid-19)	281.78 ± 58.16	505.54 ± 100.81	218.05 ± 52.77
March-May 2022 (Russian-Ukrainian conflict)	501.29 ± 15.26	721.83 ± 1.60	342.85 ± 5.35

Table 1. Mean ± standard deviation of Wheat, Soybean and Maize average prices worldwide during the indicated periods (USD per metric ton). Adapted from Nasir et al. (2022).

It is evident that the coexistence of these events, is influencing the development and productive capacity of several countries worldwide. However, following world's population demographic growth the demand for high-quality animal proteins needs to be efficiently satisfied. According to the Food and Agriculture Organization of the United Nations, eastern countries such as Pakistan and India will have overcome Europe in dairy milk production, accounting for one-third of the worldwide total milk output. In addition, the global meat production will raise by 19%, and developing countries are predicted to satisfy most of this increase (FAO, 2018). A recent report by FAO, sustained the previous observations as global increase in dairy milk was particularly evident in Asia which was the "largest milk-producing region in 2021 with a 44 percent share of the total, ahead of Europe (26 %), the Americas (22 %), Africa (5 %) and Oceania (3 %)". Moreover, the increased in meat production between 2020 and 2021 was by 4%, registered as the highest yearly increase of the 2000-2021 period. This increase was led by chicken and pig meat production which rebounded by the 11% in 2021 after the 2018 decrease caused by ASF outbreak in China (FAO, 2023). Contextually, the reduction of agricultural land availability in some regions such as Europe, calls for a more efficient use of agricultural land to sustain livestock productions. Considering all the previous observations, it is easily understandable how the demand and production

of by animal products is switching towards more both environmental and economically sustainable systems (Fig. 3).

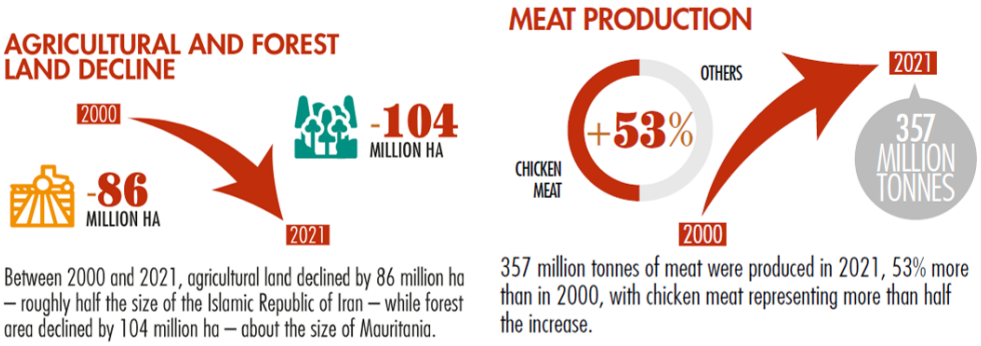

Figure 3. Agricultural and forest land decline because of both soil erosion and change of usage from 2000 to 2021 were accompanied by an increase of more efficient production systems. Adapted from FAO (2023).

Furthermore, United Nations are pursuing diversified objectives aiming to improve humans, animal and environment wellbeing. In particular, through the European Green Deal (EGD) started in 2019, United nations settled a zero-pollution ambition to be reached by 2050. In addition, one of the main EGD objectives is represented by a continuous and more efficient application of the Farm to Fork strategy (F2F), ensuring a safe, healthy and environmentally friendly food production chain. Given the importance of the feed chain within the food production system, much can be acquired from the primary sector both in terms of sustainability and public health. In this sense, sustainability aims and progresses are regularly updated by the European Feed Manufacturers' Federation (FEFAC) which settled the necessity to develop a more resilient and sustainable feed production chain. Reduction of emissions for agricultural activities and a more efficient use of primary sources (feed ingredients and water) are continuously highlighted by annual reports published by the Federation. On the other hand, the F2F strategy implemented the needs for a more sustainable production system with settling also higher standard in terms of security and health which clearly involves the feed production chain (Fetting, 2020). These measures will be further accentuated by European legislative train (within EC Green Deal schedule) to update the requirements for feed additives inclusion in the Register of Feed additives (Reg. 1831/2003/EC) which will emphasize the need to improve sustainability and reduce antibiotic usage.

1.2. Antimicrobial and metal resistance

Antimicrobial resistance (AMR) occurs when bacteria, viruses, fungi, and parasites become able to grow and proliferate in presence of pharmacological medications. Both pathogenic and non-pathogenic bacteria can acquire AMR. In particular, AMR has been indicated as the capacity of a microorganism to react towards dynamic changes in the surrounding environment, for instance the presence of toxic molecules such as antibiotics. This ability stands behind the genomic flexibility of bacteria (Martínez, 2008). This genomic adaptation can be exerted through different pathways. Firstly, the most common way for pathogens to acquire AMR genes is the plasmids-mediated pathway. Plasmids profusion in bacteria population can be facilitated non only by the vertical transmission, but also by the horizontal exchange. 3 main routes of antimicrobial-resistance genes (ARGs) transmission have been identified. Conjugation is the main route for horizontal ARGs spread (Che et al, 2021). Plasmids are characterized by varying dimensions (up to different megabases) and, consequently, by the presence of single or multiple ARGs. Therefore, ARGs transmission through conjugation is facilitated by the direct contact of the two bacterial cells. Moreover, the second considered route for plasmids among bacteria is transformation. Transformation occurs when naked DNA fragments carrying ARGs are absorbed by a bacterium subsequently to the lysis of the original bacterial cell (Vinayamohan et al, 2022). Furthermore, a third transmission route can be recognized in transduction. During transduction, bacteriophages play a key role in ARGs transmission. Specifically, bacteriophages can conserve bacterial foreign DNA in their capsid. During a subsequent cycle of infection, the bacteria host can integrate the DNA possibly carrying ARGs through homologous recombination (Zhang et al., 2011). Finally, a fourth route can be identified in natural transformation. This route occurs when bacterial cells absorb the extracellular DNA and its ARGs. Surprisingly, it has been reported that natural transformation is particularly long-lasting and can confer AMR across multiple cells generations (Dalia and Dalia, 2019).

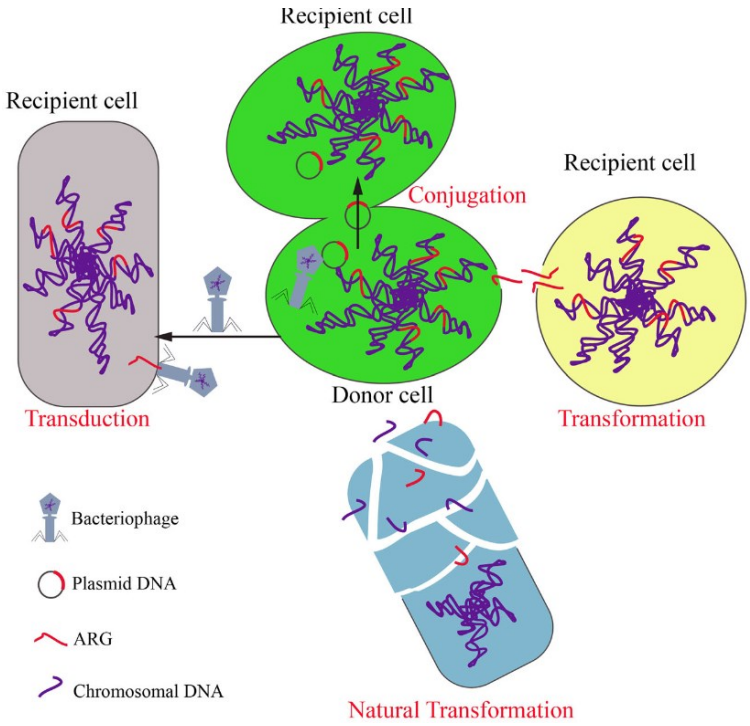

Figure 4. Main routes of ARGs transmission across bacteria (Jian et al, 2021).

Both gram-positive and gram-negative bacteria species isolates have been identified as antimicrobials resistant. Among them, *Escherichia coli* represents a bacterial specie particularly able to accumulate and diffuse ARGs. Pathogenic *Escherichia coli* strains showed resistance towards large spectrum cephalosporins, carbapenems, polymyxins and fluoroquinolones. As *Enterobacteriaceae* represents a major colonizer of piglet and calves after birth, the presence of both commensal and potentially pathogenic *E. coli* strain is particularly common. The ability of pathogenic *E. coli* in proliferating in animals enteric tract causing diarrhea strictly depends on its virulence factors, identified in the majority of cases as F4 and F18 fimbriae. In addition, *E. coli* the capacity to produce heat labile and stable toxins causing diarrhea and additional symptoms (Kolenda et al, 2015). In dairy cattle, different *E. coli* strains can cause mastitis driven by lipopolysaccharides as main virulence factor (Shpigel et al, 2008). In companion animals, *E. coli* can be responsible for upper urinary tract infections (Hutton et al, 2018). In all of these cases, depending on the severity of clinical signs, a broad range of large-spectrum antibiotics have been widely used (Poirel et al, 2018). To control gram-negatives gut proliferation and diarrhea phenomena, colistin is still one of the most used antimicrobials. In particular, sub-therapeutical dosages of colistin were commonly used to control PWD in Europe until 2006, and colistin was identified as the most prescribed in eastern countries to solve enteric diseases (Rhouma et al, 2017). Consequently, in the absence of a proper and constant control system on antibiotics administration and usage, *E. coli* resistant isolates have been identified worldwide in last years.

In livestock, especially in pig sector, following the 2006 ban on growth-promoters antibiotics the administration of high dosages of zinc and copper have been extensively considered to prevent enteric diseases. Nevertheless, the intense use of these trace elements resulted in environmental and public health issues. Specifically, Zn and Cu have been indicated as the most present heavy metal compounds in European agricultural soils in relation to manure management (Hejan et al, 2018). Indeed, Zn and Cu are commonly administered through inorganic salts such as Zn oxide and Cu sulphate characterized by very low bioavailability. In weanling piglets, zinc administration has been considered up to 3000 mg/kg of complete feed, whereas copper was administered up to 250 mg/kg of complete feed. As these trace elements are barely absorbed in animals organism, their accumulation in livestock manures and spread on agricultural soils have been facilitated (Nicholson and Chambers, 2008; Mantovi et al, 2003). Thus, it is reasonable to link the accumulation of heavy metal compounds to enhanced environmental pollution, phytotoxicity and biomagnification (Alves et al, 2016). However, dispersion of Zn and Cu in environments characterized by a dynamic interplay among diverse ecological niches does not only involve environmental issues. Zn and Cu have been linked to metal resistance genes (MRGs) and ARGs spread among diverse bacterial populations, in which co-resistance traits were also identified. As previously indicated, microorganisms actively react and easily adapt to environmental stressor. Contained trace elements concentrations are pivotal for bacterial cells maintenance, whereas pharmacological zinc and copper concentrations brings bacteriostatic and bactericidal effects. However, an excess of Zn and Cu in the environment can stimulate a selective pressure of bacterial cells as in the case of antibiotics (Seiler and Berendok, 2012). Different studies assessed that increasing dietary copper both in pigs and cattle feed can be directly correlated to the enhancement in copper-resistant isolates in fecal samples (Amachawadii et al, 2011, 2013). Pharmacological dosages of copper in piglet feed show appreciable effects in pathogens reduction. However, pathogens can adapt to extreme Cu concentration in the surrounding environment. For instance, the main mechanism proposed for which *E. coli* may resist pharmacological Cu treatments is linked to the reorganization of plasmatic outer membrane proteins in an efflux system that expel Cu ions excesses (Franke et al, 2003). Similarly, bacterial adaptation to high Zn dosages is probably driven by efflux pathways. On the other hand, Zn supplementations up to 2500 mg/kg of complete feed were linked to increased presence of AMR strains in ileum and colon of piglets when compared to not treated populations (Bednorz et al, 2013). Co-selection represents the mechanism behind the interplay between metals and antimicrobials in exerting boost effects towards bacterial AMR traits. Briefly, co-selection can occur through two distinguished sub-mechanisms: co-resistance and co-regulated cross resistance. For instance, while co-resistance involves the coexistence of ARGs and MRGs on the same plasmid, cross-resistance is based on the presence of an efflux pump mechanism that provides a simultaneous environmental resistance to antimicrobials and metal compounds (Pal et al, 2017). In addition, co-selection is driven by genes that, once expressed, are able to determine both AMR and metals efflux from the bacterial cell, resulting in a co-regulation process (Baker-Austin et al, 2006).

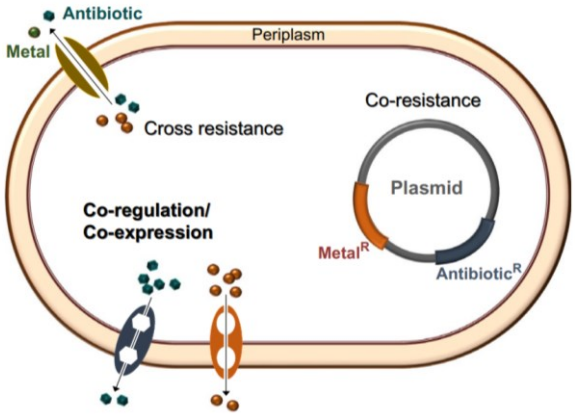

Figure 5. Co-selection pathways of ARGs and MRGs in a bacterial cell (Pal et al, 2017).

1.3. One Health approach

The economic loss related to antimicrobial resistance in Europe have been estimated around € 9 billion and linked to prolonged hospitalizations, additional pharmacological treatments and increases in healthcare costs (Prestinaci et al, 2015). In less developed countries, the absence of a proper antimicrobials prescription, regulatory and monitoring systems deeply contributes to the AMR spread (Nguyen et al, 2013). Methicillin resistance *Staphylococcus aureus* MRSA is considered a major cause of mortality worldwide, followed by multi-drug-resistant gram-negative bacteria. For instance, 4.1 % of Tuberculosis cases are considered to be multi-drug resistant and incidence of AMR Tuberculosis is predicted to increase by 2040 (Dagdostar, 2019). Furthermore, the cost in terms of lives will be of 10 million in 2050 as depicted by globally provided projections (O'Neil, 2016). In the United States, 70% of the antimicrobials are shared among human and diseases treatment (McKernan et al, 2021). European organizations have recognized the wide presence of antimicrobials in agriculture and took measures to improve the monitoring process of antimicrobials prescription. However, the increasing demand of food-producing animals in developing countries may cause difficulties in controlling antimicrobials adoption (Gilbert et al, 2021). In this context, a more efficient livestock productivity cannot exclude a continuous improvement of the "One Health" approach. The presence of AMR bacteria is ubiquitarian in various ecological niches. As diverse environments are involved in the flow of ARGs spread, an integrated and multi-sectorial approach is strictly needed. One health concept was firstly introduced in the 19th century following the first evaluations on zoonosis and their implications in human-animals relationships. To date, the One Health approach has been considered not only for monitoring but also to prevent and mitigate AMR infections within environments. Following these principles, several European organizations such as the World Organisation for Animal Health (OIE), WHO and FAO which joined in a Global Action Plan for AMR (WHO, 2015). Thus, surveillance guidelines were developed along with the establishment of a secretariat intended to promote a multi-stakeholder collaboration to contain AMR diffusion. The Global Antimicrobial Resistance Surveillance System (GLASS) born from

these jointed actions, let Europeans institutions to assess standardized procedures in order to collect, analyse, interpretate and communicating data concerning AMR (Velazquez-Meza et al, 2022). It is evident that a One Health approach must involve multidisciplinary expertise in the research field to drive a more sustainable development of the livestock sector (Mudenda et al, 2023). Until 2006, in Europe antibiotics were extensively used as growth-promoters during prophylaxis interventions to prevent diarrhea symptoms in early phases. Nevertheless, from 2006 antibiotics use has been reduced in livestock following Regulation EC 1831/2003 which depicted the ban of antibiotics as growth-promoters. Furthermore, a more conscious consideration of antibiotic drugs in livestock sector has been promoted following Regulation EU 6/2019. More recently, recognizing the role of the unconscious use of trace elements in livestock nutrition both in environmental pollution and AMR spread, European Commission promoted the ban on pharmacological dosages of zinc oxide, started in June 2022 (Bonetti et al, 2021). These interventions brought to a marked reduction of antibiotic use in Europe but not to a contextual reduction of AMR which showed a particular persistence also in those countries that early banned extensive antibiotics administration in livestock (Jensen and Hayes, 2014). Moreover, considering the lack of a proper and/or harmonized regulatory process on antibiotics usage and trace elements administration in many developing countries in food-producing animals, continuously effective and integrated measures must be applied within food-production chain. For instance, consumption of antibiotics for veterinary purposes is going to increase up to the 67% in next decades (Van Boeckel et al, 2015).

Weaning transition represents one of the most delicate phases of the cycle of production. Weanling animals are particularly susceptible to enteric diseases that may have long-term reflexes both on animal health and productivity. Gastrointestinal disorders in farm animals during critical phases represent multi-factorial pathologies that require preventions tool to avoid an excessive use of antibiotics. In piglets, one of the most common diseases is represented by post-weaning diarrhea (PWD). Furthermore, dairy calves diarrhea symptoms may characterize the first days after the separation from the dam. These disorders are driven by the proliferation of pathogenic bacteria such as *Escherichia coli* enterotoxigenic strains. Following the manifestation of severe diarrheic symptoms, antibiotics remain the only possible and efficient tool to solve enteric pathogen infection in absence of proper preventive measures. Moreover, it is quite indicative how the exposure to a single dose of antimicrobial can have long-lasting effects in terms of ARGs prevalence in fecal samples of the future herd (Zeineldin et al, 2019). Furthermore, it is clear how easily pathogens (i.e., *Staphylococcus aureus*, *Enterobacteriaceae*) can acquire AMR within food-production chain and disseminate through the personnel directly involved in animal care, slurry management and spread on agricultural soils, surface waters, biological transmission vectors and food until reaching the final consumer (Aubry-Damon et al, 2004; EFSA, 2021). Finally, it is reasonable to address nutritional management and interventions in critical phases of livestock productions a key role in controlling AMR. In particular, feed additives administration to improve gut health and enteric

microbial niches balance still represent an ideal mean for preventing the outbreaks of early-life diarrhea and, consequently, minimizing antibiotic use and AMR.

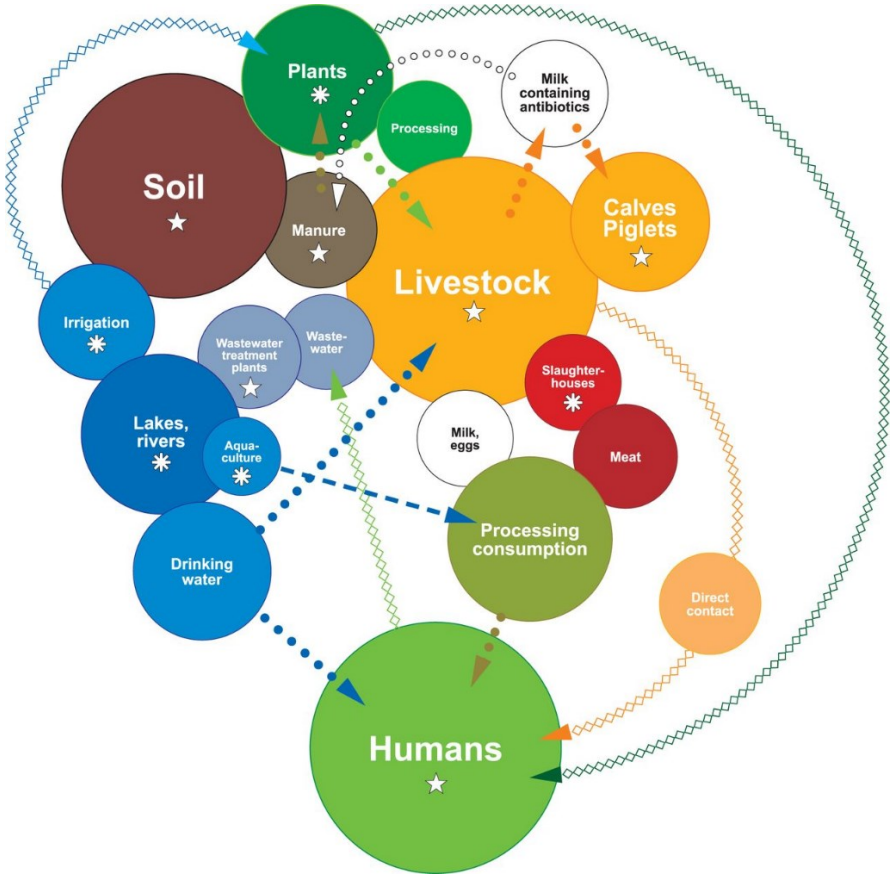

Figure 6. Diffusion routes of AMR. Stars represent the preferential reservoir for ARGs and resistant bacteria, characterized by diversified microbial niches, higher selection intensity and nutrient availability. Asterisks indicate the possible ecological niches in which resistant bacteria and ARGs can diffuse or proliferate (Thanner et al, 2016).

CHAPTER 2: The weaning process

In the present thesis, different animal models have been considered. Nevertheless, evident common traits between monogastrics and ruminants in terms of physiological changes during the weaning process have been pointed out by extensive literature. In addition, typical changes in dietary habits during this phase exert similar modulation of digestive enzymes profile, nutrients absorptive capacity and microbial niches within the gastroenteric environment when considering calves and piglets. Therefore, the following chapters will focus on the elucidation of the most critical and important changes of the weaning transition.

2.1. The Weaning transition in piglets

Weaning can be defined as a process characterized by continuous physiological changes that affect the development of the gastrointestinal tract (GIT) with long-terms reflexes on piglets health and productivity. GIT modulation starts after birth through the bioactive compounds contained in colostrum may contribute to an initial modulation of intestinal tissue through enhanced cell turn-over. This moment may firstly favor future adaptation to solid feed. However, different dietary strategies are applied during suckling period to enhance piglets weight at weaning and contain future GIT perturbances. As reported by Blavi et al. (2021) the main objectives of an efficient nutritional strategy during suckling period would be to enhance fetal growth by colostrum production and uptake while providing an ideal dietary support through creep feed administration early after birth. Nevertheless, the start of the weaning process forces a series of abrupt changes in GIT. In particular, following a first time-window characterized by a marked reduction of feed intake, enteric tissue undergoes into an involution of villi and crypts. Consequently, in the absence of proper enzymes production from microvilli brush boarders, undigested nutrients can accumulate in the distal intestine favoring the proliferation of potential pathogens and further PWD outbreak. As mentioned above, the main etiological agents promoting PWD are Enterotoxigenic Escherichia Coli strain (ETEC) expressing F4 and F18 fimbriae (Canibe et al, 2022). PWD is particularly widespread in pork production, causing profuse morbidity and mortality and loss of productivity. Intestinal barrier can be abruptly conditioned by ETEC adhesion. Once ETEC proliferates, it is quite clear how these changes can reflex marker modulations of the immune status, both locally and systemically. Thus, the intestinal barrier plays a key role in sensing infectious stimuli. Epithelial barrier cells play a key role in responding to pathogens by secreting pro-inflammatory cytokines (Pluske et al, 2018). Simultaneously, B-cells within intestinal lamina propria can be activated in an immunomodulatory process which involves also immunoglobulins release into the bloodstream (Lallès et al, 2007).

However, given the complexity of the underlying local immune and nervous system, the multi-directional responses of the intestinal barrier must be acknowledged. Specifically, both in clinical and subclinical circumstances, stress and infectious challenges can stimulate changes in GIT development by promoting the release of molecules enable to enhance intestinal permeability. For instance, Moeser et al. (2017) clearly depicted how the modulation of circulating corticotropin releasing factor (CRF) following weaning-related stressors can promote epithelial disjunctions and pro-inflammatory pathways activation by mast-cell stimulation. Consequently, due to enhance intestinal permeability the accumulation of water and electrolytes in the intestinal lumen furtherly enhances PWD symptoms while ETEC toxins have the possibility to translocate from the lumen to the circulating system.

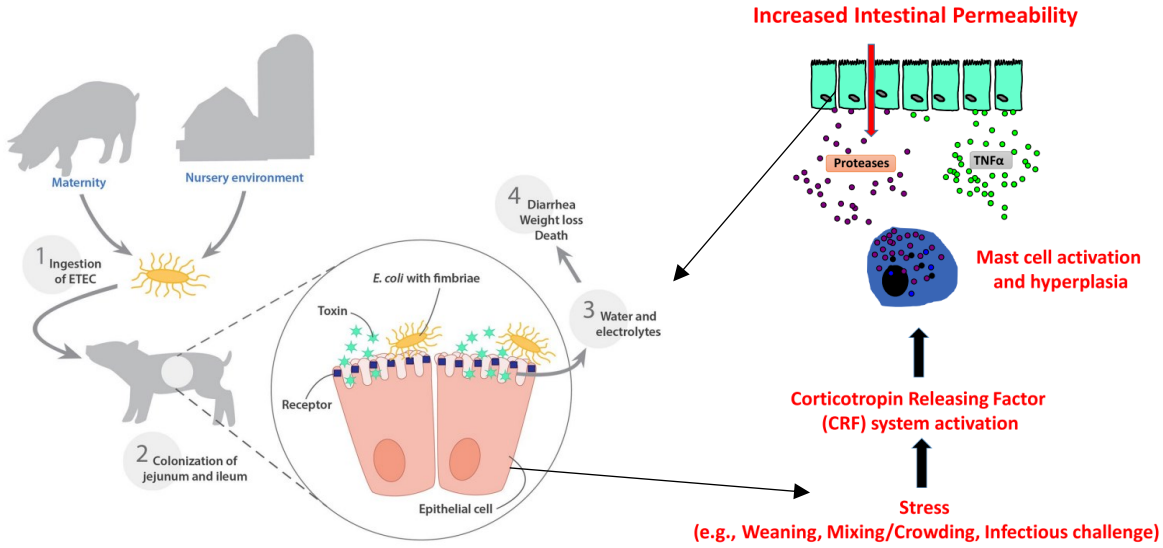

Figure 7. PWD instauration route and intestinal permeability implementation by CFR release following weaning stressors. Adapted from Rhouma et al. (2017) and Moeser et al. (2017).

2.2. The pre-weaning phase in dairy calves

The main difference between piglets and calves concerning weaning transition relies on calves pre-weaning phase. European institutions are updating scientific opinions on calves housing and welfare ideally favouring groups allocation. However, in most of European countries and United States calves are still raised under traditional rearing systems which imply individual housing (Quigley, 2024). Dairy calves are immediately separated from the dam after birth and housed individually due to a better managerial control on animal colostrum and nutrients requirements and containing pathogens infections (Mahendran et al, 2023). Dams management is pivotal and deeply influences the health of the future herd. For instance, poor nutrition management may exert future limitations in calves capacity to absorb colostrum IgG after birth and has long-lasting effects on GIT morphological development (Guy et al, 1996; da Cruz et al, 2019). Immediately after birth, adequate colostrum intake is fundamental to develop an efficiently functional GIT. During the first 4 weeks of life, milk replacer (MR) nutrients digestion and uptake are primarily regulated by abomasum and GIT which are strictly related into a neuroendocrinal regulation processes (Stahel et al, 2016). During this stage, rumen represents the 30% of the GIT total volume. Thus, it has been suggested that rumen activity assumes a primary role around 8 weeks of life (Eckert et al, 2015). In addition, during this period the increasing starch amount in diet cannot be completely fermented, resulting in sub-clinical rumen and gut acidosis that further affect GIT permeability with local and systemic inflammatory responses (Fischer et al, 2019). Therefore, similarly to monogastrics and piglet in particular, low nutrients digestion and absorption favouring pathogen proliferation and leaky gut. ETEC F5 (K99) is indicated as one of the most common etiological agents for diarrhea outbreak in dairy calves, conducting to weight loss, lethargy, anorexia and septicaemia if not adequately prevented. F5 fimbriae shows a particular affinity for glycolipids gangliosides of GIT mucosa, favouring its proliferation and its capacity to inhibit NaCl absorption, resulting in water and electrolytes accumulation in the intestinal lumen (Teneberg et al, 1994; Mirhoseini et al, 2019). Enteric diseases could influence retardation in future herd puberty age, affecting also milk production (Heinrichs, 2011). Given these considerations, it is clear how both monogastrics and ruminants share common issues concerning GIT during weaning transition. For both the categories, the onset of enteric disease is accompanied by notable changes in GIT microbiota that can have marked reflexes in its development. Considering the importance of gut microbiota and its relationship with the gastrointestinal barrier, microbiota development during weaning deserves to be elucidated in furthers chapters.

CHAPTER 3: Nutritional modulation of gut microbiota

Next-generation sequencing opened the road towards a better knowledge of gut-microbiota dynamics and their relationship with host health. Variations of gut bacterial populations during critical phases have been largely highlighted. On the other hand, the application of distinct categories of feed additives and their impact on gut microbiota have been extensively evaluated. Moreover, the interplay between the intestinal barrier and gut microbiota is increasingly gaining importance when dealing with weaning transition. The interaction between diet, intestinal barrier and microbial niches is a pillar for host health. In particular, recent studies evidenced how diversified nutritional strategies can be applied to modulate the gut microbiota gaining advantages while facing weaning transition challenges. Modulating the nutritional substrates can exert positive reflexes on gut microbial balance. Furthermore, feed additives acquired a primary role in gut microbiota modulation strategies to optimize gut health. In the present work, an integrated approach which considered gut health biomarkers and direct parameters along with microbiota variations was applied. Therefore, considering also the physiological similarities between ruminants and monogastrics when dealing with weaning transition, microbiota changes and the interplay among microbial niches and the intestinal barrier within this critical phase will be elucidated in the following chapters.

3.1. Gut Microbiota variations in piglets: from suckling period to weaning process.

Within the enteric tract, microbiota represents a key factor in the interplay among intestinal barrier regulation mechanisms. A successful weaning management involves the establishment of a balanced and healthy microbiota. Gut microbiota is pivotal for the maintenance of gut health and for key functions related to diversified pathways. Enteric ecological niches are involved in nutrient digestion, absorption and endogenous biosynthesis of molecules enable to contribute to gut tissue development. On the other hand, it is well known that several niches actively influence the immune response of the animal. The modulation of piglets gut microbiota during weaning have been lengthily evaluated. Nevertheless, it is important to clarify that the first microbial GIT colonization is pivotal for an ideal future gut development and for host health and performances. The initial GIT microbial pattern is particularly linked to the establishment of adult-like ecological niches. Immediately after birth, the gut environment is characterized by an extensive presence of oxygen. Therefore, the first colonizers, mainly bacteria belonging to *Firmicutes* and *Proteobacteria* phyla (*Bacteroides, Escherichia-Shigella, Clostridium sensu stricto 1,* and *Fusobacterium genera*) contribute to the establishment of an anaerobic environment that furtherly condition the development of a dynamically diversified microbial population (Chen et al, 2018). Nevertheless, it must be considered that most of these genera are represented by potential opportunistic bacteria and their constant presence should be not overlooked. Abrupt changes in the initial microbial pattern can already have long-lasting effects on animal health. *Escherichia-Shigella* have been indicated as a pathogen positively correlated with pro-inflammatory cytokines whereas *Fusobacterium*

have been pointed out as potential pathogens that could drive initial diarrhea phenomena in suckling piglets (Han et al, 2022; Hermann-Bank et al, 2015). Interestingly, *Fusobacterium* have been related to severe haemorrhagic diarrhea in dogs and ulcerative colitis in humans (Suchodolski et al, 2012; Rajilić-Stojanović et al, 2013). In addition, *Clostridium difficile* proliferation in suckling piglets GIT have been widely associated with neonatal diarrhea (Keel and Songer, 2006).

Much of the changes during the first days of life involve several environmental factors and are also common among diversified models, even humans. The first contact with the sow colostrum/milk uptake drives much of the changes in gut microbiota composition during the days of life. Therefore, *Bacteroides*, *Lactobacillus* and *Bifidobacteria* became increasingly represented within the first week of life. It is reasonable to link these changes both to a direct transfer of bacteria from the sow to piglets, especially in the case of *Lactobacillus* whereas *Bacteroides* abundance can be influenced by the increasing presence of oligosaccharides abundantly present in sow milk and milk replacers (Bian et al, 2016; Li et al, 2012). In addition, direct contact with maternal fecal material is fundamental for early microbiota development, as along with milk may exert further reflexes in terms of differential regional expression of intestinal immune and functional genes (Liu et al, 2019). The importance of dietary glycans of milk origin in modulating gut microbiota have been extensively evaluated, and it must be related also with the enzymatic and metabolic capability towards glycans in depicted in young animals. As indicated by Zivkovic et al. (2013) considering a human model, glycans can determine a milk-oriented microbiota (MOM) supported by both enzymatic and metabolic pathways. From the first to the third week of life, *Enterobacteriaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Clostridiaceae* and *Lactobacillaceae* abundances increasingly raise in suckling piglets gut (Frese et al, 2015). Another factor to consider when discussing microbiota variations in suckling piglets gut is the effect of the birth weight. Several studies showed how low-birth weight piglets (LBW, from 0.75 to 0.95 kg) evidenced specific gut microbiota patterns (i.e. lower abundance of *Lactobacillaceae*, *Ruminococcaceae*, *Prevotellaceae* and increased presence of *Fusobacterium*) that can possibly have long-terms effects on animal health and performance (Trevisi et al, 2023). Changes in dietary habits markedly influences gut microbiota composition. It would be reasonable to speculate that creep feeding may play a key role in starting a nutritional modulation toward weaning. However, there is a lack of studies concerning creep feeding and microbiota variations as the available literature mostly focuses on the weaning phase (de Vries and Smidt, 2020). On the other hand, it has been demonstrated how MR composition and colostrum intake can affect gut microbiota modulation (Correa et al, 2023). Nevertheless, much of the changes associated with transition from liquid to solid feed happen when completely interrupting MR administration. Immediately after weaning, gut eubiosis is abruptly lost due to the transient anorexia that previously discussed. Normally, a safe weaning transition would involve a gradual decrease of *Proteobacteria* and *Fusobacteria* with a contextual increase of *Firmicutes* and *Bacteroidetes* as commonly most represented phyla in piglets gut after weaning (Hu et al, 2016). More in detail,

opportunistic bacteria (*Escherichia-Shigella*, *Helicobacter*, *Salmonella*, *Vibrio* and others) should gradually decrease after weaning, whereas genera linked to better vegetal carbohydrates digestion and endogenous VFAs production should gradually increase (*Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae*). As much of the changes in microbiota composition are driven by the available substrate, it reasonable to link the increasingly abundancies of amylolytic and VFAs producers to changes in dietary formulas. During weaning the passage from pre-starter to starter feed is accompanied by a reduction of milk-derived product such as whey. Contextually, *Prevotella* and *Paraprevotella* abundances enhance due to changes in the protein substrates. These modulations were confirmed by Chen et al. (2017), nevertheless authors outlined that gut microbial stability can be reached around 10 d post weaning due to the possible prolonged permanence of appreciable abundances of opportunistic bacteria waiting the chance to proliferate. However, the key to contain their proliferation is the increasing diversity during weaning, as enhanced biodiversity is strongly associated with a more resilient and adult-like gut microbiota (Fassarella et al, 2021). More specifically, it is reasonable to link the decrease in biodiversity with the lower competitive capacity of commensal niches towards opportunistic pathogens. In any case, the onset of post-weaning diarrhea in this window of time can be evidenced by clear and abnormal microbiota changes. A first signal can be identified in a change of *Firmicutes* and *Bacteroidetes* abundances. Whereas a healthy microbiota should display a higher abundance of *Firmicutes* and *Bacteroidetes*, an inverted trend accompanied by loss of diversity is typically correlated with enhanced diarrhea probability. *Bacteroidetes* have been indicated as pivotal proteolytic bacteria, and their reduction can be linked to increases of *Proteobacteria* and pathogenic *Enterobacteriaceae* resulting in PWD onset (Zheng et al, 2023). More in detail, the excess of undigested proteins that can accumulate as substrate in the distal enteric tract may favour *Proteobacteria* proliferation and excessive biogenic amines production which further contribute to intestinal inflammation and permeability increase. Typically, these events are accompanied by the reduction of *Prevotellaceae* both in hindgut and fecal samples whereas *Escherichia-Shigella* abundance enhances.

3.2. Gut microbiota development in pre-weaning calves

Similarly to piglets, immediately after birth calves GIT is colonized by bacteria, fungi, archaea derived from maternal and environmental contact. GIT microbiota development in pre-weaning calves is strongly conditioned by the increasing restriction of milk replacer consumption, which represents a common practice to stimulate rumen functionality. It must be considered that despite the increasing activity of the rumen during this phase, intestine represents the major site of absorption for most of nutrients and VFAs. However, dairy calf enteric microbiota modulation during the pre-weaning period was less frequently evaluated. Nevertheless, the idea that dynamic changes in gut microbiota may have reflexes on future herd productivity enhanced the attention toward gut microbiota shifts in dairy calves. A first imprint of future microbiota pattern is given by the dam. For instance, in-utero colonization of the gut through the meconium has been associated to the future shedding of *Faecalibacterium*,

Bacteroides, *Lactobacillus*, and *Butyriccoccus* (Alipour et al, 2018). Considering the initial dietary regime, starting microbiota pattern of dairy calves is largely similar to suckling piglets. While feeding colostrum and transition milk, facultative anaerobes such as *Enterobacteriaceae* (*Escherichia coli*) are predominant and persists until the end of the first week of life, as in the case of *Lactobacillus* spp., *Bifidobacterium* and *Faecalibacterium* (Song et al, 2018). Furthermore, also *Actinobacteria* have been found particularly present in neonatal calves microbiota and have been related to high quality colostrum uptake (Van Hese et al, 2022). Interestingly, Actinobacteria presence immediately after birth has been related to better gut barrier integrity, lower gut permeability and inflammation (Messman and Lemley, 2023). Subsequently, with increasing anaerobe conditions, phyla such as *Firmicutes*, *Bacteroidetes* and *Proteobacteria* actively colonize the enteric tract. *Proteobacteria* are particularly present early after birth but tend to decrease in correspondence to enhanced starch assumption. Therefore, *Firmicutes* and *Bacteroidetes* became the most representatives gut bacteria. More in detail, it has been pointed out that in calves considering the gradual change in dietary habits and enhancing concentrate intake favour the establishment of predominant taxa such as *Bulleida* and *Turicibacter* in the hindgut whereas *Paraprevotella* and *Prevotella* became particularly abundant in distal GIT tracts (Dias et al, 2021). It must be considered that much of the starch post-ruminal digestion and absorption largely depends on microbial fermentation already during the first weeks of life. in addition, gene sequences strictly related to amino acids and energy metabolism were detected in gut microbiota of dairy calves. Therefore, a direct correlation between gut bacterial colonization and initial growth has been pointed out (Elolimy et al, 2020). Dysbiosis may occur during the first days of calves life. A loss of microbiota richness and diversity can be detected approximately around 7 d of life and may have long-terms reflexes (Wilkins et al, 2019). A return to facultative anaerobes higher abundances is detected during dysbiosis and neonatal diarrhea. Therefore, whereas genera such as *Lachnospiraceae* and *Ruminococcaceae* or resilient *Faecalibacterium* associated with carbohydrates metabolism and gut health decreases, *Lactobacillus* spp., *Streptococcus* and *Escherichia coli* enhance. This modulation is mainly drive by enhanced intestinal permeability and oxygen tension. Contextually, *Lactobacillaceae* increase is caused by D and L-lactate production in gut lumen (Gomez et al, 2022). The accumulation of lactate contributes to damages in the osmotic balance of the gut lumen which furtherly accumulates water, electrolytes and undigested nutrients that favour pathogens proliferation. Therefore, the dynamics of *E.coli* or other pathogens (i.e. *Salmonella typhimurium* and *Cryptosporidium parvum*) outburst in post-calving dairy ruminants are very similar to weanling piglets (Jessop et al, 2024). Nevertheless, to better understand the mechanisms behind diarrhea onset and microbiota variations the interplay between gut microbiota and intestinal barrier deserves to be highlighted in next sub-chapter.

3.3. An overview of gut-barrier and microbiota interplay

The crosstalk between dysbiotic microbiota and the intestinal barrier has been previously evaluated. Much of this interplay is driven by nutritional substrates metabolism by products. Especially after ETEC infections, the presence of dysbiotic ecological niches have marked reflexes in terms of barrier and host immune response modulation. This crosstalk is defined by bilateral characteristics. The presence of mucosal receptors activates the direct response of the intestinal barrier during pathogens proliferation. In particular, the mucosal barrier is characterized by the presence of secretory IgA (SIgAs) which provide a local screening tool for both beneficial and pathogenic bacteria. IgA are not transferred through placenta in the considered species. Thus, an efficient transfer through colostrum deeply contribute to the establishment of effective local immunity protection in the enteric tract. From one side, the capacity to recognized antigens let SIgAs to establish a surrounding coat for commensal bacteria (i.e. *Bacteroides*) regulating their distribution and mucous colonization along the gut lumen while on the other they contribute to activate the immune response toward potential pathogens (Takeuchi and Ohno, 2021). This process of stimulation of the "exogenous" microbiota is pivotal in newborn animals. In addition, it has been indicated that specific miRNAs regulating positively correlated with *Bifidobacterium* and *Lactobacillus* spp. have been identified. More in detail, these molecules can be directly transferred through colostrum intake and regulate gut microbial colonization and immune system development (Du et al, 2023). Reasonably, the barrier-microbiota crosstalk can be deeply conditioned by the presence of proliferating pathogens. Firstly, to proliferate in the intestinal lumen ETEC needs to influence the anti-inflammatory response in intestinal mucosa. For instance, by reducing the expression of TLR5 factor which have the role to recognize ETEC virulence factors (F4, F18) starting the anti-inflammatory response (Bin et al, 2018). Moreover, the presence of gram-negative pathogens lipopolysaccharides (LPS) in the pathogens' outer membrane exacerbates the intestinal tissue pro-inflammatory response furtherly increasing gut permeability (Suzuki, 2013). However, digestive disorders onset is not exclusively related to ETEC or other pathogens proliferation. As previously stated, *Proteobacteria* play a key role in relation di gut barrier status. Their metabolic activity is clearly mainly focused on amino acids. From this point of view, the production of contained quantities of biogenic amines can be useful to promote gut barrier development, however excesses may promote gut inflammation and permeability (Özogul and Hamed, 2018). A comparable mechanism can be linked to *Fusobacteria* catabolic activity which is linked to amino acids. Reasonable concentrations of amino acids in the intestinal lumen promotes hydrogen sulfide (H_2S) production derived from *Fusobacteria*. Hydrogen sulfide can contribute to intestinal permeability maintenance, nevertheless, H2S excesses exert toxic effects on the intestinal barrier (Ma et al, 2022). Moreover, in relation to amino acid metabolism, the role of indole as signal molecule that drive intestinal microbiota structure is pivotal. Indole can both directly promote intestinal barrier functions or determine changes in bacteria structures. Briefly, indole can regulate the expression of virulence factor whereas once metabolized by commensals

influences the expression of glucagon-like-peptide 1 displaying metabolism regulation potential (Chimerel et al, 2014). Nevertheless, indole can serve as an intercellular signal molecule originated from tryptophan metabolism. Indole can regulate both the expression of virulence factors in *E. coli* or have detrimental effects on plasmids whereas indole-derived metabolites may exert positive immune modulation acting on enteric T-cells through *Lactobacillus* spp. (Lee and Lee, 2010; Cervantes-Barragan et al, 2017). In addition, indole is involved in metabolic pathways related to endogenous amino acids biosynthesis by useful genera belonging to *Bacteroides* or *Parabacteroides* phyla possibly improving gut mucosa development (Liang et al, 2018). On the other hand, the role of commensal bacteria in regulating and maintaining the intestinal barrier functions have been evaluated in different animal and human models. Briefly, *Bifidobacteria* structures, identified in fimbriae and extracellular polysaccharides (EPS), can modulate the local immune response of the host. Among *Bacteroidetes*, diversified species are able to reduce the pro-inflammatory response and modulate phagocytosis regulating macrophages differentiation (Hiippala et al, 2018). Furthermore, fermentation activity of commensal bacteria modulated through dietary fibre is a key factor for gut health regulation. For instance, *Bifidobacteria* fermentations contributes to protect the host from *E. coli* infections by producing acetate which provides both tissue antibacterial activity along with tissue development and protection (Fukuda et al, 2011). Acetate, mostly derived from pectin fermentation have been pointed out as an important fermentation by-product that trigger a reactive IgA production from specific commensal bacteria (*Enterobacterales*) contributing to microbiota eubiosis (Takeuchi et al, 2021). Other commensals such as *Roseburia* spp. and *Lachnospiraceae* have a diverse fermentative activity that can produce other short chain fatty acids (SCFAs), such as butyrate and propionate, which contribute to gut homeostasis and decrease proinflammatory cytokines activity (Tamanai-Shacoori et al, 2017; Hoffman et al, 2016; Che et al, 2019). In addition, butyrate may modulate macrophages differentiation and serves as energy source for enterocytes. Interestingly, butyrate has been related to the oxygen-status of in intestinal barrier which represents one of the main factors involved in increased permeability during bowel inflammation (Bortoluzzi et al, 2022). On the other hand, propionate derived from the fermentative activity of *Firmicutes*-belonging genera such as *Pediococcus* can alleviate the inflammatory response by reducing the expression of TNF-α in the intestine (Sun et al, 2020). Finally, valerate and branched-chain fatty acids (BCFAs) such as isovalerate and isobutyrate derived from polypeptides and saccharides fermentations are frequently linked to modulation of helpful microbiota niches such as *Lachnospiraceae*, *Ruminococcaceae*, *Lactobacillus* spp. and *Clostridium* spp. genera. Interestingly, it has been pointed out that antibiotic administration negatively condition microbiota shifts towards lesser SCFAs and BCFAs production, possibly affecting gut permeability (Che et al, 2021). From these considerations it is clear how nutritional regulation can exert marked reflexes in microbiota-host relationship during critical phases. Therefore, modelling the diet, for instance varying fibre and protein sources, can be useful to favour the biosynthesis of pillar molecules for a positive gut barrier-

microbiota interplay. In these terms, the administration of feed additives can represent an ideal strategy to shape gut microbiota composition and its biological interactions.

3.4. Feeding the gut microbiota: strategies during critical phases

3.4.1. Proteins

Dietary proteins have been pointed out as the main nutrients involved in gut functions impairments during weaning transition, especially in monogastrics. As previously stated, excess of nutrients, and proteins in particular, can be detrimental as may favour the proliferation of pathogenic *Enterobacteriaceae* and harmful *Proteobacteria*. Therefore, modulating dietary protein have been evaluated as a strategy to improve gut health. Vegetal proteins have been associated in the past to enhanced temporal sensibility to PWD due to the presence of antigens in soybean meal (glycinin and β-conglycinin). In addition, during weaning transition there is an evident passage from protein sources that are easily digestible to vegetal proteins which requires a different enzymatic pattern. Therefore, in the past it has been reported that protein digestibility may condition the severity of PWD phenomena. For example, when comparing a control group fed pea protein with a second one fed plasma during E.coli experimental challenge, PWD symptoms were more frequent in the control group (Owusu-Asiedu et al, 2023). Furthermore, excesses of undigested proteins may favour *Proteobacteria* proliferation and, consequently, the production of biogenic amines from amino acids decarboxylation may influence intestinal barrier functions and pathogens proliferation (Rist et al, 2013). Nevertheless, more recently feeding strategies have been applied to contain PWD. For instance, protein restriction has been considered in several countries to minimize PWD in piglets. First, the possibility to face poor performance following excessive protein constraint has been pointed out. Nevertheless, limiting the protein restriction within the first 14 days after weaning may exert positive reflexes on microbiota composition and activate a further compensatory growth regulated by diversified metabolic pathways. As indicated by Hou et al. (2021) a basal diet characterized by a 16% of crude protein (CP) can favour the abundances of positive *Parabacteroides*, *Butyriccoccus* and *Succinivibrio* which are related to SCFAs production, hemicellulose, and xylan degradation. Protein restriction increases bacterial diversity and richness, favouring ammonia reduction in distal digesta while increasing the presence of *Faecalibacterium* and *Lachnospiraceae* (Tian et al, 2019). As confirmed by further studies, this strategy is useful also in enhancing barrier integrity by conditioning tight junctions (TJ) expression bringing contextual positive modulation of *Ruminococcaceae*, *Blautia* and *Prevotellaceae* abundances in colon digesta (Wang et al, 2022). Therefore, both the modulation of the protein source and temporary restriction may represent an ideal strategy to promote positive variations in gut microbial niches while gaining positive reflexes on the intestinal barrier, especially in monogastrics. Nevertheless, this approach may deserve to be better investigated in dairy calves dietary regime. Moreover, much of the interest in varying protein sources to modulate gut microbial niches come also from the diversified composition in terms of structural fibre which will be further introduced.

3.4.2. Fibre

It is well known how dietary fibres (DF) can actively modulate intestinal microbiota. However, recent research is constantly focusing on the effects of DF modulation on the interplay between gut microbiota and health. DFs are composed by non-starch polysaccharides (NSPs) and lignin which represent the insoluble fraction, and a soluble component represented by neutral detergent fibre (NDF). While soluble fibres are more fermentable, insoluble fibres are generally more difficult to ferment in the intestinal lumen. Therefore, the interest in DFs increased due to their presence in all of the nutrient substrates (protein, energy and fibre sources) which may strongly condition gut microbiota and health during weaning transition when modulated. Given their structural complexity, DF are one of the most selective substrates toward bacterial populations. In particular, this selectiveness depends on how much the insoluble fraction is represented (Hu et al, 2022). For instance, in piglets, *Bacteroidetes* are strongly conditioned by the CF dietary content. Furthermore, *Bacteroidetes* abundance is positively conditioned by rapeseed meal supplementation more than soybean meal. Rapeseed is characterized by a higher insoluble DF fraction than soybean meal. Thus, an excess of rapeseed meal administration may change the balance among *Bacteroidetes* and *Firmicutes* negatively influencing piglets performances (Ellner et al, 2022). Nevertheless, moderate concentrations of insoluble fibres can be helpful in containing diarrhea during weaning transition (Canibe et al, 2022). On the other hand, modulating DF favoring the soluble fraction, NDF in particular, may exert positive reflexes such as enhancing *Bifidobacteria* and *Lactobacillus* spp. along the enteric tract. A practical example was given by Chen et al. (2014): by substituting soy-derived fibre with a combination of wheat bran and pea-fibre in weanling piglets the authors enhanced *Lactobacillus* spp. and *Bifidobacteria* abundances in piglets gut. In any case, excesses of soluble s must be avoided within the first two weeks after weaning, as in case of ongoing diarrhea soluble fibres can exert detrimental effects (Tang et al, 2024). Therefore, the prebiotic characteristics of soluble fibres may contribute to enhance the competitiveness of commensal and beneficial bacteria toward potential pathogens. Nevertheless, reflexes in terms of gut barrier-microbiota crosstalk have been recently considered when dealing with DF variations. In particular, DF has been widely associated with VFAs production in the gut lumen. Endogenous SCFAs and butyric acid in particular serve as primary energy sources for enterocytes replication, gut development and mucin production. However, most of the time their role in conditioning local and systemic immunity is overlooked. More specifically, by modulating DF, SCFAs can influence adaptive immunity and inflammatory response by enhancing immunoglobulins production, directly influencing macrophages maturation in lamina propria and producing beneficial peptides (Majii et al, 2018).

3.4.3. Starch

Among nutritional components, starch is one of the main involved in gut microbiota regulation and functions in pre-weaning calves. Interestingly, it was shown how changing the balance of CP and starch in dairy calves diet can deeply influence distal gut microbiota and relative microbiota pathways (Chen et al, 2023). From one side, an adequate amount of starch is necessary to bring butyrate production and, consequently, gut development. Nevertheless, excesses of undigested starch may promote pathogens proliferation. Interestingly, it was shown that microbial fermentations in small intestine represent the major pathways to contribute to starch degradation rather than enzymatic reactions (Gilbert et al, 2015). Therefore, excess of starch promotes a surplus in terms of butyrate production, which further contribute to decrease intestinal pH and DFs digestibility due to reduced fibrolytic bacteria presence (Júnior and Bittar, 2021). in pre-weaning calves, varying DF by administering different sources gave notable results in terms of fecal microbiota composition. Kodithuwakku et al. (2021) showed that 100 g/day of timothy hay and psyllium during pre-weaning period increased *Lactobacillaceae* and *Bifidobacteria* but also *Prevotellaceae* during the first 3 weeks of life, bringing appreciable propionate production and enhanced growth performance. Therefore, it is important to consider NSPs from forages as a useful tool to contain the excessive fermentation of available starch contained in starter feed. In addition, a good strategy to further stimulate a balance among beneficial bacterial community is to consider the supplementation of a contained quantity of forages and starter feed from the very first days of life. For instance, combining alfalfa hay, starter feed and milk replacer stimulated fibrolytic and amylolytic bacteria proliferation in the cecum of yak calves promoting immune homeostasis (Wu et al, 2021).

CHAPTER 4: Feed additives to promote gut health

Regulation 1831/2003/EC provide practical information regarding the feed additives available on the market is. The document, commonly referred to as "European Register of Feed Additives", indicates a clear categorization of feed additives. In particular, additives are divided into the following categories within the Annex I of the Regulation:

- Technological additives: added to feeds for technological purposes (i.e. emulsifiers added to feed as pelleting coadjutant).
- Sensory additives: added in order to improve the organoleptic properties of feeds (i.e. flavoring compounds).
- Nutritional additives: added to feed to satisfy the specific nutrient requirements.
- Zootechnical additive: used to improve animal performance, health and favorably affect the environment.
- Coccidiostats and histomonostats.

In order to be included in the Register, feed additives undergo to a preliminary evaluation performed by the European Food Safety Authority (EFSA) which provides a scientific opinion that must be adopted by the European Commission briefly after publication. This process involves a series of *in vivo* trial to assess the safety and efficacy of the feed additives proposed by an applicant (i.e. industrial stakeholders). Therefore, EFSA is responsible for risk assessment whereas the European Commission provides the risk management. On the other hand, as indicated by the Annex II of Reg. 1831/2003/EC the Joint Research Center (JRC) is responsible for the preservation of samples and for the evaluation of the analytical chosen during the procedures of approval. Regulations and guidelines of the workflow related to feed additives approval are continuously updated to encounter also to satisfy the increasing requirements of compounds not only able to ameliorate health and welfare of livestock but also to promote sustainability. Therefore, there is an increasing interest in the market in improving feed additives features. Livestock diets are modulated to ameliorate economic sustainability and animal performance. Diverse typologies of compounds may contribute to increase diet quality, reducing costs and improve gut health. Essential oils and derived phytochemicals are one of the most important categories of feed additives thanks to the multitude of beneficial effects that exert on host health. Similarly, organic acids are key compounds in guaranteeing the stability of the gut environment. Contextually, the interest in the concept of "blended compounds" raised both commercially and scientifically: if less recently research was more focused on acknowledging the effects and modes of action of single molecules, nowadays the possible synergisms originated by diversified combinations on gut health are of particular interest. Furthermore, whereas more bioavailable alternatives to pharmacological zinc and copper have been extensively evaluated, the combination of more of these alternatives need to be further assessed. Finally, Probiotics represent one

of the most valuable classes of compounds that enable to ameliorate gut health. However, new solutions and strains are increasingly investigated. Therefore, in the next chapters the compounds object of the manuscripts presented in this thesis will be highlighted.

4.1. Essential oils and phenolic compounds

Essential oils and phenolic compounds represent one of the most diversified categories of molecules due to the very diversified chemical structure that can be found in nature. Essential oils are mixtures of volatile biochemical compounds characterized by immunomodulatory, anti-inflammatory and bactericidal properties. Specifically, the lipophilic properties of these compounds enhance their capacity to break down bacterial cytoplasmatic membranes and inner structures (Burt, 2004). Essential oils (EOs) are composed by secondary metabolites categorized in terpenes and phenolic compounds (i.e., carvacrol, thymol, eugenol and tannins), which have been widely appointed as the principal sources of essential oils growth-promoting properties. Nevertheless, essential oils are characterized by a notable variability in terms of composition which may affect their effectiveness in terms of gut health. EOs chemical extraction requires a large amount of carbon dioxide and water are used. Interestingly, innovative extraction methodologies such as microwave or fluid extraction have indicated as the best alternatives in terms of energy, water and EOs yield capacity, contributing to a more sustainable EOs production (Aziz et al, 2018). Nevertheless, geographical variability, environmental conditions and soil chemical properties may determine significative differences in terms of EOs composition (Steiner and Syed, 2015). Therefore, direct chemical synthesis of phenolic compounds and technologies such as EOs encapsulation are increasingly gaining importance to obtain standardized feed additives, both in terms of chemical composition and biological feedback. Phenolic compounds are represented by a series of molecules that vary from simple monoterpenes or phenolic molecules to highly polymerized compounds characterized by the presence of methyl esters (Vuolo et al, 2019). Typically, EOs and phenolic compounds are link to growth promoting properties. Increased body weight, average daily weight gain and feed conversion ratio were registered in pigs and chicken following a multitude of studies (Mahfuz et al, 2021). In addition, this mode of action may derive from a better nutrient digestion as both EOs and phenolic compounds are able to stimulate gastric and enteric endogenous enzymes secretion while increasing the absorptive capacity of intestinal villi (Zeng et al 2015). Therefore, the intestinal permeability can also be positively conditioned by EOs and phenolic compounds. Indeed, their anti-inflammatory and antioxidant properties may have direct reflexes on gut tissue integrity. When supplementing EOs and phenolic compounds decrease ROS, TBARS and pro-inflammatory cytokines was observed. In addition, the immunomodulatory capabilities of these molecules may condition lymphocytes proliferation (Nehme et al, 2021). By reducing pro-inflammatory cytokines and exerting antioxidant effects, these molecules can positively affect barrier integrity (Valdivieso-Ugarte et al, 2019). On of the most considered compound among these categories is carvacrol (Bonetti et al, 2021).

4.1.1. Carvacrol

Carvacrol is a monoterpene compound found in several varieties of oregano, thyme and savory. Interestingly, it was shown that carvacrol is characterized by a fast metabolization process as it is quickly absorbed in the small intestine after ingestion, whereas the 29% of ingested carvacrol is degraded into distal enteric tracts (Michiels et al, 2008). Furthermore, thanks to his hydrophobic properties, carvacrol is characterized by a notable antimicrobial activity, which is accompanied by the scavenging potential towards gram-positive and gram-negative pathogens (Alagawany et al, 2015). These modes of actions are linked to immunomodulatory processes when considering the dietary supplementation of natural extracts or EOs containing carvacrol (Stelter et al, 2013). Finally, the capacity to stimulate endogenous digestive secretion can directly act on feed intake and characterize an overall positive modulation of livestock growth performance (Hashemipour et al, 2013). Moreover, an increasing interest is raising toward the possible synergisms among phytogenic compounds and terpenes. Interestingly, Rebucci et al. (2022) outlined the potential of blended carvacrol, thymol, and cinnamaldehyde in maintaining the antioxidant balance and reducing fecal calprotectin over the weaning period in piglets, evidencing a protective effect on the intestinal barrier. Furthermore, in general these compounds can maintain the ecological balance among gut microbial niches promoting beneficial bacteria, preventing pathogens proliferation, diarrhea occurrence and containing amines production. Furthermore, these blended compounds frequently displayed gut microbiota modulation, exerting also positive effects on the intestinal barrier (Mo et al, 2023). Nevertheless, as depicted by Luise et al. (2023) different combinations of EOs and their combination with organic acids maybe characterized by diversified feedbacks in terms of gut microbiota modulations and gut health parameters, therefore it will be important to continue evaluating different solutions in further studies. Therefore, following this necessity, the first research project of the present thesis was performed.

4.1.2. Tannins

Among polyphenols compounds tannins acquired a particular interest in livestock nutrition thanks to their astringent properties capable to effectively preventing diarrhea during weaning transition. Tannins derives from *Castanea sativa* Mill. or *Vitis vinifera* varieties. Interestingly, their administration is limited to contained concentrations due to possible negative side-effects linked to nutrients digestion (proteins in particular) and, consequently, reduced feed intake (Bee et al, 2016). Tannins are particularly effective against gram-positive bacteria. Tannins are able to limit substrate availability for pathogens replication, inhibiting extracellular microbial enzymes and mitochondrial oxidative phosphorylation or damaging membrane structures causing cell death (Liu et al, 2013). On the other hand, tannins have appreciable effects towards pro-inflammatory and pro-oxidant mediators, especially those characterized

by the higher number of hydroxyl groups (Ricci et al, 2016). In ruminants, the use of forages particularly rich in condensed tannins can have positive reflexes in terms of fecal Escherichia coli shedding reduction and also prevent multifactorial diseases such as frothy bloat thanks to protein-binding capacity (Berard et al, 2009; Sottie et al, 2014). In general, tannins revealed positive modulation of GIT microbiota by enhancing the abundancies of genera such as *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillus* thanks to oligomers that can affect microbial niches by producing functional compounds (Huang et al, 2018).

4.2. Organic acids

Organic acids (OAs) have been extensively used during critical phases to enhance animals gut health. One of the first mode of action evaluated regarding organic acids administration was the acidification of the gastric environment to facilitate protein digestion and reducing the accumulation of undigested nutrients in distal GIT tracts. Indeed, hydrochloric acid (HCL) production is extremely limited immediately after weaning due to the buffering capacity of many feed components such as minerals sources and vegetal proteins. For instance, soybean-based based components are administered up to the 30% in weanling piglets but are characterized by high acid binding capacity (ABC). On the other hand, calcium carbonate, limestone and zinc oxide are characterized by the highest ABC values (He et al, 2022). More specifically, the passage from milk-based proteins to vegetal ones requires lower enteric pH values to enhance pepsin enzymatic activity, reaching the maximum efficiency at values lower than 4 (Suiryanrayna and Ramana, 2015). Furthermore, organic acids are characterized by diverse constants of dissociation (pKa) which define their bactericidal and bacteriostatic capability. In particular, pKa indicates the pH value in which to acid compound goes into dissociation. Therefore, lower pKa values characterize stronger acids (Wang et al, 2014). It must be outlined that undissociated OAs may enter pathogens cytoplasm thanks to their lipophilic characteristics and, encountering higher pH values, dissociate into protons and ions, altering internal pH and promoting cell leakage of gram-negative bacteria (Greene et al, 2021). Therefore, the lipophilic nature of OAs may also condition their effectiveness. For instance, gram-positive bacteria such as *Streptococcus spp.* and *Clostridium perfrigens* are more susceptible to MCFAs whereas SCFAs displayed higher effectiveness toward gram-negative pathogens such as *E. coli*, *Campylobacter jejuni* and *Salmonella spp.* The reason of these differences stands behind the higher lipophilic nature of MCFAs and the presence of bacterial LPS in gram-negative wall which confer resistance towards MCFAs (Tugnoli et al, 2020).

4.2.1. Medium and Short chain fatty acids

MCFAs and SCFAs have been extensively evaluated as effective tools to face weaning transition. SCFAs are saturated fatty acids characterized by a maximum of 5 carbon atoms within the molecular structure, whereas MCFAs are composed from 6 to 12 carbon atoms. In both cases, the administration of the glycerides forms allows them to efficiently null their low palatability and quick absorption in the gastric environment. Therefore, mono-or try- glycerides and saponified forms favor a slower release of the active compounds from the stomach to the small intestine (Rebucci et al, 2021). MCFAs can represent a valid energy substrate for cellular turn-over, contributing to the integrity of the intestinal tissue and also promoting the endogenous secretion of cholecystokinin, affecting feed intake and overall performance (Zentek et al, 2011). MCFAs can influence the immune status of the animal by directly acting on T cells lymphocytes through specific signal pathways activation (Zhang et al, 2018). On the other hand, it has been shown that MCFAs may reduce pro-inflammatory cytokines. Following *in vitro* studies, caprylic (C8), capric (C10), and lauric (C12) acids have shown to markedly improve the immune response of porcine intestinal cell line (Martínez-Vallespín et al, 2016). MCFAs revealed positive effects on intestinal microbiota of weanling piglets. Butyrate supplementation displayed effective anti-inflammatory reflexes by reducing decrease malondialdehyde (MDA), tumor necrosis factor a (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), reducing intestinal permeability and positively conditioning tight junctions expression in monogastrics animals (Huang et al, 2015; Zou et al, 2019). On the other hand, SCFAs sources such as tributyrin and sodium butyrate have been efficiently used to face neonatal diarrhea in dairy calves. In particular, butyrate can be useful in anticipating rumen development and further promote calves performances. In dairy female calves, tributyrin can promote the development of gut tissue by enhancing intestinal villi length, bringing better nutrient absorption, and reducing diarrhea incidence within the first 4 weeks of life (Gorka et al, 2011, 2014). In addition, different studies highlight consistent data regarding the effects of butyric acids on dairy calves GIT microbiota. In particular, carbohydrates, starch utilizers and VFAs producers *Lachnospiraceae*, *Ruminococcaceae* and *Rikenellaceae* along with bacteria related to immune regulation and amino acids digestion such as *Prevotella* are enhanced by butyric acid administration as tributyrin or sodium butyrate (Liu et al, 2022). Moreover, Hanczakowska (2017) discussed how MCFAs administration in weanling piglets diet can affect the intestinal flora by reducing *E. coli* and *Clostridiaceae* counts. Given the wide range of available molecules within OAs compound group, the possibility to gain synergistic effects through different combinations is increasingly explored in livestock nutrition research field. Interestingly, from a technological point of view, physiochemical features of glycerides enhanced the interest in developing new solutions in terms of coated mixture of organic acids alone or in combination with essential oils or probiotics (Jackman et al, 2020).

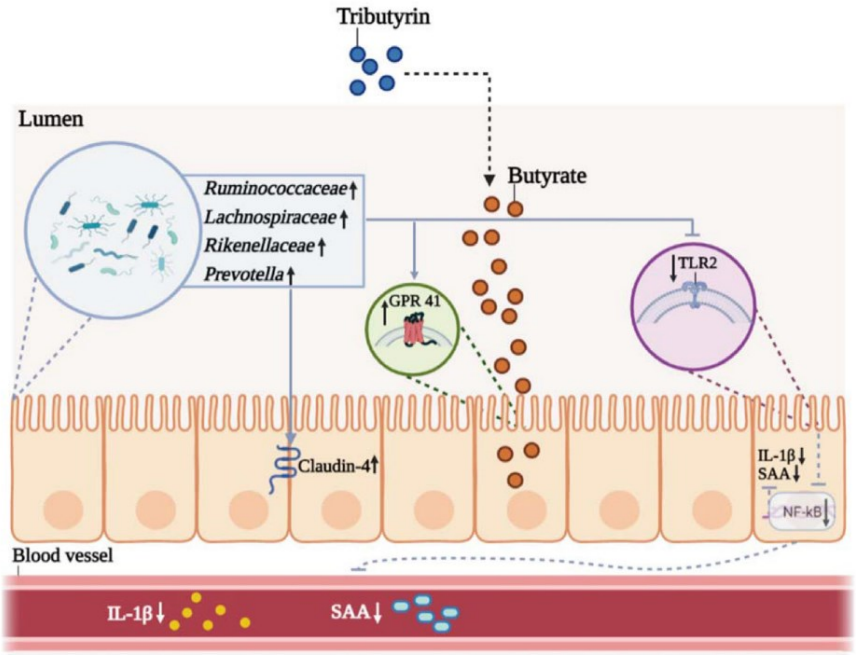

Figure 8. Graphical representation of tributyrin modes of actions in GIT. TLR2: toll-like receptor 2; IL-1b: interleukin 1b; GPR 41: G-protein coupled receptor 41; SAA: serum amyloid A; nuclear factor kappa B: NF-kB. Adapted from Liu et al. (2022).

4.3. Zinc and copper

Zinc and copper acquired a primary role among trace elements when dealing with gut health. The importance of trace elements is given by their biological role as co-factor in numerous enzymatic reactions. From one side, trace elements supplementation needs to meet the requirements to maintain biological functions, whereas over-nutritional dosages, specially of zinc and copper, have been used to promote gut health and growth. Most of zinc homeostasis occurs in the small intestine, where it is absorbed as divalent cation. Then, zinc can be bounded to metallothioneins or enter in a labile form into enterocytes cytosol and bind to ZIP family transporters. These linkages regulates both zinc homeostasis and diversified enzymatic reactions. However, zinc homeostasis maintenance is also regulated by endogenous zinc secretions, which enhances in case of deficiency, and by the presence of others trace elements divalent cation, such as copper, cadmium, and iron (Krebs, 2000; Windish, 2002; Brugger and Windish, 2017). Historically, inorganic forms of Zn (oxide, sulphate or carbonate) are preferred as feed supplements, nevertheless, it must be pointed out that more recently both organic and inorganic formulations characterized by a higher bioavailability are available on the market. Zinc supplementation have been extensively considered to prevent diarrhea in young monogastrics and ruminants. The positive effects exerted by zinc supplementation on host intestinal tracts and gut microbiota have been widely link to better growth performance in monogastrics and ruminants species. Intestinal morphology is

clearly favoured by zinc supplementation, bringing higher enzymatic activity (especially for α-amylase, trypsin, lipase and chymotrypsin) and, consequently, lower probability of enteric disorders (De Grande et al, 2020; Satessa et al, 2020; Xia et al, 2017). Supplementing zinc over the nutritional requirements can exert notable effects in terms of immunity, as zinc is involved in numerous regulatory processes for B and T-cells development, which further affects circulating immunoglobulins levels (Wessels et al, 2021). However, precise mode of actions still needs to be clarified (Broom et al, 2021). As reported by Duffy et al (2023), zinc binding proteins are pivotal for the constitution of gut microbiome, as up to the 20% of the dietary zinc can be utilized by host enteric bacterial niches. In addition, appreciable changes in terms of *Enterobacteriaceae*, *Clostridiaceae* and *Lactobacilli* have been found by administering Zn to piglets and calves, and several authors pointed out the capacity of zinc to promote competitive exclusion of beneficial bacteria towards *E. coli* (Shannon and Hill, 2019). Nevertheless, most of these results were characterized by a dose-dependent effectiveness, which in the past favoured pharmacological dosages of zinc oxide. However, the recent legislative changes which promoted ban on pharmacological zinc-based medications for oral supplementation opened the road to extended research on more bioavailable molecules. Interestingly, Chang et al. (2023) highlighted the possibility to shape both microbiota composition in terms of diversity indexes and beneficial bacteria abundancies (*Bacteroides*, *Faecalibacterium*, *Lactobacillus*) and immune parameters as well (IgM and IgM) by supplementing Zn-methionine. Similar findings were also pointed out in weanling piglets studies when considering inorganic and organic zinc sources, which favoured performances, gut health, and beneficial microbiota variations (Luise et al, 2024; Diao et al, 2021). Similarly to Zn, copper has been widely supplied in feeds to promote growth performance and gut health, especially considering copper sulphate ($CuSO_4$) administration up to 250 mg/kg of complete feed. Cu is mostly absorbed in stomach and duodenum as in free ions form and through a transcellular saturable process and/or through tight junctions pores (van den Berghe and Klomp, 2009). Copper homeostasis is regulated by specific transporters and chaperone proteins. Specifically, in case of copper deficiency, Cu-ATPase pump system activates an increasing efflux of Cu ions from the basolateral membranes to blood stream, whereas in case of adequate copper resources, the synthesis of metallothioneins able to bind Cu in the liver enhances (Toriumi et al, 2005). Furthermore, Cu displayed to promote lipids digestion lipogenesis, fatty acids uptake and lipids mobilization from peripheral tissues (Espinosa et al, 2020). Copper acquired a particular importance thanks to his bactericidal properties, pointed out as main mode of action in over nutritional Cu supplementations. Indeed, Cu supplementation revealed positive effects in term of humoral immune response because of pathogen proliferation containment in the intestinal tissue (Espinosa and Stein, 2021). Interestingly, copper has been associated to varying effects in term of gut microbiota modulation depending on the source. From the evidences collected by Broom et al. (2021) Cu supplementation may condition potential pathogens such as *Enterobacteriaceae*, *Fusobacterium* and *Escherichia* whereas these changes can be associated to reduced pro-inflammatory cytokines production. Zn and Cu ions shared pathway related to metallothioneins and recent research studies

pointed out that varying dietary zinc and copper ratios in livestock can exert reflexes in terms of trace elements bioavailability. In particular, the recent tendence is to produce “micronized” or “porous” particles characterized by a lower surface which results in higher availability of the active compounds in the molecule (Scott et al, 2018; Lei et al, 2022). For these reasons, a second research project was carried out to link the relationship between diverse zinc and copper ratios to different feedbacks in terms of gut health.

4.4. Probiotics

Probiotics are live organisms supplied to balance host gut microbiota and health, promoting growth performance and immune defences. Generally, probiotics represents a natural approach to improve host health and have been widely pointed out as valid substances to prevent antibiotic usage. Lactic acid producing bacteria (LAB) represents the most considered probiotic compounds in livestock nutrition. This large group of microorganisms is composed of gram-positive facultative aerobe or anaerobe bacteria (cocci or rod-shaped bacteria), yeast and fungi (Bhogoju and Nahashon, 2022). The motivations behind the great extent of probiotics usage lays behind their wide range of beneficial reflexes on the host. According to Anee et al. (2021) the main modes of action of probiotics can be classified as follows: a) Establishing balance among microbial niches along the entire GIT and, consequently, enhancing the competitivity of commensal bacteria towards pathogens adhesion; b) production of functional molecules such as bacteriocins and defensins; c) fortification of intestinal barrier functions; d) reduction of intestinal pH; e) Systemic and local immunity regulation by directly acting on lamina propria immune cells. Among LAB, *Lactobacilli* and *Bifidobacteria* are still the main used probiotics along with *Saccharomyces*, *Lactococcus* and *Streptococcus*, whereas diverse *Bacillus* strains are acquiring importance. Probiotics can be helpful also in stimulating the synthesis of endogenous nutrients (i.e. vitamins) and enzymes, contributing also to the production of VFAs further affecting gut development and pathogens proliferation (Plaza-Diaz et al, 2019). On the other, probiotics can contribute to enhance protein and lipids digestion which can be helpful in ameliorating gut health status in different models as discussed above (Ushakova et al, 2015). *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus* and several other strains have been showed to efficiently obstruct GIT colonization by directly acting on pathogens through bacteriocins. Essentially, bacteriocins can pass the bacterial membrane of diverse gram-negative pathogens blocking DNA synthesis in the cytosolic environment and promoting cell leakage. In addition, probiotics can directly act for competitive exclusion with pathogens while decreasing the luminal pH which furtherly favour the production of other antibacterial compounds such as acetic acid (van Zyl et al, 2020). Probiotics compound, as in the case of *Lactobacillus casei* and *Lactobacillus rhamnosus*, can directly communicate with toll-like receptors (TLR) to promote tight junctions expression in the small intestine, favouring gut barrier integrity. For instance, *Lactobacillus plantarum*, was shown to reallocate zonulin-1 and occludine by interacting with TLR2 receptors whereas *Lactobacillus rhamnosus* was found particularly active in promoting claudin-3 expression

through TLR4 (Karczewski et al, 2010; Segers et al, 2014). Interestingly, it has been speculated that the capacity of probiotics to modulate the intestinal barrier may depend on specific probiotics components (i.e. peptidoglycans or proteins) rather than specific bacterial strains (Rose et al, 2021). Probiotics can cross the lamina propria to directly act on immune cells. In the small intestine, contextually to their interaction with TLR pathways, *Bifidobacteria* and *Lactobacillus* are able to modulate TLR6 and decrease pro-inflammatory cytokines expression. Furthermore, in different animal models it was demonstrated that T and B cells can be stimulated to enhanced proliferation by diverse probiotic strains, which also promotes the reduction of creatine kinase and alkaline phosphatase remarking enterocytes apoptosis reduction (Ding et al, 2021). Probiotics are widely recognized as efficient gut microbiota modulators. *Lactobacillus plantarum* increased diversity and richness along with the abundancies of *Erysipelotrichaceae*, *Spirochetaceae*, *Ruminococcaceae* families in weanling piglets gut microbiota (Shin et al, 2019). Oral administration of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* can enhance the abundances of beneficial genera such as *Prevotella*, *Ruminococcus* and *Streptococcus* in jejunum and cecum of weanling piglets, increasing also nitrogen metabolism positively influencing skeletal muscles development pathways (He et al, 2023). *Bifidobacterium animalis* displayed positive microbiota variation in weanling piglets ileal digesta by decreasing Escherichia-shigella and Helicobacter abundances and promoting growth performance (Pang et al, 2022). As previously anticipated, these typologies of variations may contain diarrheal disorder which is a priority also in dairy calves management. Indeed, *Lactobacillus* spp. dietary administration in pre-weaned dairy calves increased fecal *Akkermansia* and *Bifidobacteria*, linked to intestinal barrier integrity and immune response respectively (Fernández-Ciganda et al, 2022). In addition, the interest toward probiotics mixture to gain multiple effects on gut health have been extensively considered. For instance, mixture of lactic acid bacteria has been found to deeply condition gut microbiota composition by enhancing the abundances of beneficial bacteria such as *Ruminococcaceae* and *Lachnospiraceae* whereas adding yeast to the mixture revealed *Bifidobacteria* enhancement (Liu et al, 2022). However, in most of the cases LAB bacteria are poorly resilient in the gastro-intestinal tract, resulting in possible biases in terms of gut health and microbiota responses. Nevertheless, *Bacillus* spp. spores have been found particularly resistant in harsh environments characterized by low pH and can be supplemented into probiotic mixtures possibly boosting their effectiveness. In addition, *Bacillus* strains have been found to exert similar reflexes in comparison to LAB on host health in monogastrics but have been poorly applied in dairy calves nutrition. From this point of view, the necessity to evaluate the effectiveness of newly solutions was addressed in the third paper of the present thesis.

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CHAPTER 5: Aim of the project

The aim of the following project was to evaluate different nutritional strategies based on feed additives to face weaning transition challenges in monogastrics and ruminants, with a particular focus on gut health. To perform this project, three *in vivo* trials were conducted. Given the similarities among the species considered during weaning transition, the specific intent was to apply a comprehensive strategy involving the evaluation of direct and undirect biomarkers of intestinal health, focusing also on gut microbiota variations. The choice of the additives compounds was based on the idea to evaluate the synergistic effects of different combinations of molecules (phytochemicals and MCFAs or potentiated zinc with monovalent copper) and solutions which show a great potential but lack of consistent evaluations in the literature, as in the case of the tested *Weizmannia faecalis* strain. Contextually to my project, the possibility to evaluate alternative strategies to enhance gut health of weanling piglets through a Secretory IgA stimulating vaccine was explored. In particular, part of the activity conducted at the department of Animal and Veterinary Sciences of Aarhus University (Denmark) are reported in a fourth section. Therefore, the following pages will be divided into 4 different chapters dedicated to 3 papers completed and submitted during my PhD pathway and a brief report highlighting the experience gained during the abroad secondment. In particular, in **chapter 6**, a first published study will elucidate the effects of blended compounds (carvacrol, tannic acid and MCFAs administered at 1500 mg/kg) on gut health of weanling piglets raised in a commercial farm. The necessity to further optimize trace elements supplementation to enhance gut health in weanling piglets diet was furtherly evaluated in a second submitted study reported in **chapter 7**. In this case, specialty oxide sources characterized by higher bioavailability (potentiated zinc and monovalent copper oxide) were administered in 3 different ratios (within European and non-European levels of inclusion) and compared to a group fed a pharmacological dosage (2500 mg/kg) of classic ZnO formulation. The possibility to implement probiotic-based strategies to enhance gut health of pre-weaning dairy calves was the aim of a third study discussed in **chapter 8**. In particular, a group of 10 calves were fed with basal diet supplemented with *Weizmannia faecalis* DSM32016, not yet been approved for dairy ruminants, and compared to a control group. In addition, alternative strategies to enhance gut health based on vaccines have extensively developed in last years. On of the main quests in vaccination strategies optimization in the future will be to stimulate a local gut mucosa immunization through IgA secretion stimulation by parenteral vaccination. Therefore, this possibility was evaluated during an abroad secondment in which a parenteral vaccine developed to counterattack piglets post-weaning diarrhea was tested on *E. coli* (F4/F18) challenged animals, as depicted by **chapter 9**.

CHAPTER 6: Dietary supplementation with a blend composed of carvacrol, tannic acid derived from *Castanea sativa* and *Glycyrrhiza glabra*, and glycerides of medium chain fatty acids for weanling piglets raised in commercial farm

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Brief introduction to the study: My first step into animal nutrition research field is represented by a previous published study focused on blended carvacrol, thymol and cinnamaldehyde administration to enhance gut health of weanling piglets as project for my MSc degreed in Animal Husbandry sciences and Technologies (https://doi.org/10.1016/j.livsci.2022.104959). Therefore, the first part of my PhD project was dedicated to a continuation in the study of the application of blended compounds in animal nutrition. The data presented derived from the necessity to evaluate the prevention of piglets post-weaning diarrhea considering blended compounds, as, nowadays, feed additives synergisms may represent an ideal tool to enhance piglets gut health. The application of ensembles of diversified molecules able to condition the overall GIT health is increasingly gaining interest, especially after the ban of pharmacological ZnO. Nevertheless, in most cases, the synergistic effects of different compounds lack of elucidation. Thus, we decided to perform an *in vitro* evaluation of the tested blend, followed by an *in vivo* trial performed in a local commercial farm just after the ban on pharmacological ZnO was applied. To date, the performed work brought to my first presentation in a national conference at Animal Science Association congress helded in Monopoli (Italy, 13-16 June 2023) and to an open access publication on Veterinary research communications journal, which is possible to view at: https://doi.org/10.1007/s11259-024-10539-1.

Abstract

This study aimed to evaluate the dietary administration of a blend composed of carvacrol, tannic acid derived from *castanea sativa* mill and *Glycyrrhiza glabra*, medium chain fatty acids (MCFAs) glycerides for weanling piglets. An *in vitro* digestion followed by total phenolic content (TPC) and total antioxidant activity (TAC) assessment was performed before the *in vivo* application. At weaning, a total of 210 piglets were randomly allocated to two experimental treatments (7 replicates/15 piglets for each

replicate). Control group (CTR) was fed a standard basal diet while the treated group (T) was fed the basal diet mixed with 1500 mg/kg of blend. After *in vitro* digestion, TPC and TAC evidenced peaks at the end of oral and gastric phases in comparison to the intestinal one in line with the high content of phenolic compound (<0.05). Treatment conditioned body weight and average daily gain (P<0.05), fecal score on 6, 7, and 8 d after weaning (P<0.05). At 35d, the T group showed a decrease in salivary cortisol compared to CTR (P<0.05). Duodenum and jejunum sections of T piglets revealed higher villi (P<0.05), deeper crypts (P<0.01), and increased V/C ratio (P<0.01). CTR showed a higher expression of duodenal Occludin (P<0.05). Jejunal E-cadherin and Occludin were more expressed in T jejunum sections (P<0.05). Twelve differentially abundant genera were identified in T group cecal samples. Potentially harmful *Clostridium sensu stricto 13* was reduced by the treatment (P<0.05). In conclusion, the tested blend positively affected salivary stress markers and the gut health of weaned piglets.

Keywords: Feed Additive , Microbiota , Gut health, Weaning

Introduction

Weaning is a process accompanied by notable changes in intestinal morphology, especially regarding villus height and crypt depth, caused by transient anorexia (Lallès et al, 2007). Moreover, the impairment in terms of digestive enzymes production at this stage contributes to promoting the accumulation of unabsorbed nutrients in the large intestine where potential pathogenic bacteria such as enterotoxigenic strains of *Escherichia coli* could promote the onset of the post-weaning diarrhea (PWD) (Fairbrother et al, 2005). Furthermore, this threat is exacerbated by the lack of an adequate immune status in weanling piglets, which potentially undergo chronic and acute inflammatory status (Pié et al, 2004). Consequently, PWD represents the most harmful condition during the weaning transition of piglets promoting a detrimental situation in economic terms, characterized by high morbidity and mortality, veterinary interventions, labour costs and negative reflexes on productive parameters (Laird et al, 2021). After the European Community ban on antimicrobials as growth promoters in 2006, pharmacological dosages (2000-3000 mg/kg of complete feed) of Zinc Oxide (ZnO) represented a widely diffuse strategy to promote gut health in weanling piglets by avoiding PWD (Corino et al, 2021). However, starting from June 2022 the European Commission decided to ban the prescription of ZnO oral medication for livestock (Bonetti et al, 2021). The concerns that led to the ban of high dosages of ZnO in feed were linked to the low bioavailability of this trace element and, consequently, to its environmental impact, but also to the co-selection of antibiotic-resistance bacterial strains (Mantovi et al, 2003). Therefore, the research of valid alternatives able to promote gut health without having negative effects in terms of pollution or safety is strictly needed.

Active compounds from natural extracts could block the activation of both inflammation and oxidative stress signal pathways (Galli et al, 2020; Na and Surh, 2008). In this sense, one of the most studied compounds is carvacrol which has a large spectrum of antimicrobial activities against gram-negative

and gram-positive bacteria (Roller and Seedhar, 2002). Briefly, this antimicrobial activity is determined by the presence of the hydroxyl group in the molecule of the natural compound, which contributes to the release of bacterial lipopolysaccharides (LPS) from the Gram-negative membrane. Furthermore, among polyphenolic compounds, tannic acid (TA) proved to be useful in positively modulating the intestinal microbiota, improving energy metabolism through higher production of volatile fatty acids (VFA), and increasing the integrity of the intestinal barrier (Song et al, 2021). Moreover, the administration of medium and short-chain fatty acids (MCFAs) could also represent a valid tool for enhancing gut health during the weaning transition (Chen et al, 2019). Indeed, it is recognized how molecules like lauric acid (C:12), capric acid (C:10), and caprylic acid (C:8) could inactivate the bacteria proliferation both by promoting the acidification of the intestinal environment or acting against the expression of virulence factors. In addition, the inclusion of low dietary levels of MCFAs showed modulatory effects on the enteric microbiota population (Omonijo et al, 2018) and demonstrated positive reflexes in terms of gut morphology on villus height, and tight junctions proteins (TJs) in the proximal tract of the small intestine (Zentek et al, 2011). Moreover, the administration of glycerides of fatty acids has been reported to control pathogens proliferation and reduce post-weaning diarrhea (Correa et al, 2021). Therefore, despite widely diffused knowledge on the effects of tannic acid, MCFAs and carvacrol (Song et al, 2021; Lauridsen, 2020), little is known about the possible effects on the gut health of piglets when fed a blend obtained by these single active compounds. The critical aspect of blends is probably linked to the possible interaction between the different components that may affect their efficacy in conditioning the gut environment (Canibe et al, 2022). However, different composition blends exerted positive reflexes on the gut health of weanling piglets raised in experimental facilities (Rebucci et al, 2022; Luise et al, 2023). Nevertheless, testing these blends in commercial farm conditions could reveal different effects and new insights. Indeed, the exposure to typical stressors of the weaning transition combined with the higher density of commercial farms could make piglets more easily vulnerable to PWD. For these reasons, the study aim was to investigate the effects of the dietary administration of a blend composed of carvacrol, TA and MCFAs on the gut health of weanling piglets raised in commercial farm conditions.

Materials and methods

In vitro digestion, Total phenolic content and antioxidant capacity.

In vitro digestion was performed as reported by Regmi et al. (2009) with minor modifications introduced by Lanzoni et al. (2023). At the end of each digestion step, aliquots (about 1 mL) were taken and frozen immediately at -20°C and used to measure total phenolic content (TPC) and antioxidant activity. In parallel, at the end of digestion, the samples were filtered using paper filters (Whatman 54 Florham Park, NJ), thus obtaining the undigested fraction (UF). Subsequently, the filters were dried overnight at 65 °C. Then, the dry matter digestibility (% dry matter; DM) was measured using the following formula:

$$DM\ Digestibility = \left[\frac{DMx - DMy}{DMx}\right] * 100$$

Where DMx is the sample dry matter percentage, while DMy represents UF dry matter percentage.

For TPC, the protocol of Attard (2013) was used with minor adaptations as reported by Lanzoni et al. (2023). Tannic acid, methanol, Folin–Ciocalteu (FC) reagent, and sodium carbonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid was prepared in a 1:2 dilution. FC reagent was diluted with distilled water (1:10); contextually 1 M solution of sodium carbonate was prepared. Then, 100 µL of each sample (7 aliquots) was added to 500 µL of FC and 400 µL of sodium carbonate and incubated at room temperature for 20 min. At the end of the incubation period, samples were read at 630 nm. TPC Values were expressed in terms of tannic acid equivalent (mg TAE/100 g of dried samples). The FRAP assay was performed following the protocol of Abdelaleem and Elbassiony (2020), with minor modifications. FRAP values are expressed as mg $FeSO_4$/100g of dried sample. The antioxidant activity was assessed using the ABTS method according to the protocol of Re et al. (1999) with minor adaptation. For TPC, FRAP and ABTS oral, gastric and intestinal phase were considered. Values were expressed in terms of Trolox equivalent (mg TE/100 g of dried samples). Analyses were performed on biological and technical triplicate for each parameter.

Animals housing and experimental design

The *in vivo* trial was performed at Azienda Agricola Pianoverde of Santorelli-Brontesi S.S., Boarini Farm, Via Cascina, 25023 Leno (Brescia). At weaning, corresponding to 0 d of the trial, 210 cross-bred twenty-eight-day old piglets (Stambo HBI X Dalland 40) were randomly distributed, according to their body weight, into the treatment and control experimental groups (105 each). Each group was replicated seven times, with 15 piglets per pen forming the experimental unit. Animals were housed in two different rooms of 7 replicates each and 1 pen at disposal as infirmary. Trial lasted 35 days corresponding to 61 d old piglets. Each pen had a slatted floor and was fitted with a stainless-steel feeder and nipple waterers. The rooms were lit by a combination of daylight and artificial light. Rooms temperature, humidity, and air quality were automatically controlled. Ventilation was achieved by two, variable-speed fans linked to temperature sensors. The temperature inside the building was approximately 28 °C at the start of the

trial and was adjusted weekly until a final temperature of 24–25 °C was achieved. The relative humidity was settled between 60–70%. Piglets had water and feed available *ad libitum*.

The control group (CTR) was fed a basal diet, whereas the treated group (T) was fed the basal diet mixed with a dosage of the product corresponding to 1.5 kg/ton. For the treated diet, the proper quantity of additive was weighed using a balance and premixed with a small amount of the feed as a carrier, before adding this to the final mix to ensure homogeneous distribution in the complete feed. All diets were formulated to meet or exceed the nutrient requirements recommended by the NRC (2012) for post-weaning piglets (Table 1). The treatment compound was a blend of 5% of carvacrol, 23% of monoglycerides, diglycerides and triglycerides of medium chain fatty acids (capric, caprylic and lauric acid) and tannic acid derived from 26% of *castanea sativa* mill and 2% of G*lycyrrhiza glabra* extract stabilized on silica (Gastroherb Plus produced by Phytosolutions, Caldes de Montbui, Barcelona, Spain).

Table 1-Composition and chemical analysis of the post-weaning diets (% on dry matter basis). DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fibre; Ca: calcium; P: phosphorus; NE: net energy.

Ingredients, % as fed	Pre starter (0-14 d)	Starter (14-35 d)
Barley	19.50	18.50
Corn	24.00	24.00
Wheat	9.00	10.00
Dry whey	5.00	4.00
Soybean oil	2.00	2.00
Fish meal	2.00	-
Bakery by-products (10% CF)	10.00	8.00
Soybean meal 48 % CP	6.50	8.50
Extruded soybean	4.00	6.00
Toasted soybean	3.75	3.75
Vitamin and mineral premix[1]	0.25	0.25
Flacked barley	3.00	3.00
Soybean concentrated	2.00	2.00
Soybean oil	1.00	1.00
Calcium carbonate	1.20	1.20

Monocalcium phosphate	0.60	0.60
Sodium chloride	0.20	0.20
Bran	4.00	5.00
Beet pulp	2.00	2.00
Chemical analysis, % DM	Pre starter (0-14 d)	Starter (14-35 d)
DM, %	88.00	88.00
CP, %	17.46	17.65
EE, %	6.57	6.61
CF, %	3.23	3.42
Ash, %	5.72	5.55
Ca, %	0.89	0.83
P, %	0.55	0.53
NE, kcal/kg	2.471	2.469
Lysine, %	1.20	1.20
Methionine, %	0.43	0.42
Methionine + Cysteine, %	0.72	0.72
Threonine, %	0.78	0.78
Tryptophane, %	0.23	0.23

[1]Additives (per Kg): Vitamin.pro-vitamin and analogue substances:3a672a Vitamin A 63.193 UI3a671 Vitamin D3 13.226 UI3a700 Vitamin E (All-rac-alfa-tocoferile acetate) 353 mg3a711 Vitamin K3 14.7 mg3a821 Vitamin B1 14.7 mg3a825ii Vitamin B2 47.0 mg3a831 Vitamin B6 14.7 mg Vitamin B12 0.29 mg3a314 Niacin 294 mg3a841 Calcium D-pantothenate 245 mg3a316 Folic Acid. 5.1 mg3a880 Biotin 0.59 mg3a890 Choline Chloride 903 mg Trace elements :E4 Copper (3b412 Copper oxide [I].) 476 mg E4 Copper (3b405 Copper sulphate[II] pentahydrate.) 113 mg E1 Iron (3b103 Iron sulphate [II] monohydrate.) 1.344 mg E 2 Iodine (3b202 Calcium iodate anhydrous.) 11.2 mg E5 Manganese (3b502 Manganese oxide [II].) 560 mg E 8 Selenium (3b801 Sodium selenite.) 2.8 mg E6 Zinc (3b603 Zinc oxide.) 504 mg Preservatives: E330 Citric acid 1.1 mg1a297 Fumaric acid 5.300 mg Antioxidant: E310 Propyl gallate 0.37 mg E321 Butylhydroxytoluene (BHT) 118 mg Binders: E551a Silicic acid 2.5 mg E563 Sepiolite 5.491 mg Digestion promoters:4a16 6-phytate (EC 3.1.3.26) 2.118 OTU4a1617 Endo-1.4-beta-xilanase EC 3.2.1.8 19.980 EP

Growth parameters, fecal score and general health

The body weight and feed consumption were measured at 0, 14, and 35 days. Feed was distributed daily in trails after being weighed by a scale and having registered the weighted quantity. Therefore, average daily feed intake (ADFI) and average daily gain (ADG) were calculated for the 3 different periods of the trial (0-14 d, 14-35 d, and 0-35 d). Feed conversion rate (FCR) and feed efficiency (FE) were subsequently obtained by ADFI/ADG and ADG/ADFI ratios respectively. Body weight (BW) and ADG were also registered accounting for single animals. Mortality, pathologies, or unusual adverse events were recorded daily. Fecal score evaluation was performed daily from 0 d to 35 d on trial through a 0 to 4 scale (0=normal stool, 4=diarrhea) as reported by Ruckman et al (2020).

Salivary cortisol level, immunoglobulins A (IgAs), and total antioxidant capacity (TAC)

Saliva samples were taken on days 14, 21, and 35 of the trial using Salivette® tubes (Sarstedt AG& Co., Germany) from one subject per replicate. A cotton swab was kept in the mouth of the animal for 1–2 min following the procedures described by Escribano et al. (2019). The cotton swab was then placed in the tube and centrifuged at 3000 rpm for 13 min. The saliva aliquots obtained by centrifugation were then stored in Eppendorf tubes (Sarstedt AG& Co., Germany) and frozen at − 80° C until analysis.

Cortisol and IgAs were quantified using competitive and sandwich ELISA kit tests according to the manufacturer's instructions (Immunological sciences, Società Italiana Chimici, Rome, IT). IgAs and Cortisol values are expressed in ng/ml. The total antioxidant capacity (TAC) evaluation was performed through ferric reducing antioxidant power (FRAP) method performed with a commercial kit (Elabscience Biotechnology Co.,Ltd). Values of FRAP are reported as µmol/l of Trolox equivalents.

Intestine Histology and Histometry

At the end of the trial, animals were slaughtered, and intestinal tissue sampling was performed (n=7 per group). Portions of 1 cm^3 of the small intestine (duodenum 2 cm after the pylorus and duodenojejunal junction, according to Ishida et al. 2018) were immediately collected and fixed in 10% neutral buffered formalin for 24 h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. Microtome sections (4 µm thick) of both duodenum and jejunum were stained with Hematoxylin–Eosin (HE) to establish structural details. On these HE-stained sections, the height of intestinal villi (V) (10 villi measured per section) and the depth of intestinal crypts (C) (10 crypts measured per section) were measured and calculated by image analysis software (Proview, Optika, Italy). The ratio between villi and crypts (V/C) was also calculated.

Gut barrier assessment: E-Cadherin, Zonulin-1, and Occludin immunofluorescence staining

Other sections of the duodenum and jejunum were used for immunofluorescence. Briefly, after rehydration, heat-induced antigen retrieval was performed (citrate buffer pH 6, 5 minutes microwaves 600 W, followed by cooling, twice). After washing three times in Phosphate buffer saline (PBS, pH 7.4), treatment with the Avidin–Biotin blocking kit solution (Vector Laboratories Inc., Burlingame, CA USA) was performed. Sections were incubated with the primary antiserum: anti-E-Cadherin (E-CAD; 1:30, ab15148, Abcam, UK), zonulin-1 antibody (ZO-1, 1:100, Cat. No. GTX108592, GeneTex, USA), and anti-Occludin antibody (1:100, ab216327, Abcam, UK), for 24 hours at room temperature and washed in PBS. Afterward, sections were incubated with 10 µg/ml goat biotinylated anti-rabbit IgG (Vector Laboratories Inc., Newark, USA) for 2 hrs at room temperature. After rinsing twice in PBS, the sections were treated with Fluorescein–Avidin D (Vector Laboratories Inc., Newark, USA), 10 µg/ml in NaHCO3, 0.1 M, pH 8.5, 0.15 M NaCl for 2 hrs at room temperature. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium with DAPI (SKU H-1200-10, Vector Laboratories Inc., Newark, USA) and observed using a Confocal Laser Scanning Microscope (FluoView FV300; Olympus). The immunoreactive structures were excited using Argon/ Helio–Neon–Green lasers with excitation and barrier filters set for rhodamine. Images containing superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel. The absence of cross-reactivity with the secondary antibody was verified by omitting the primary antibody during the first incubation step.

For the quantification of each of the three immunofluorescence, duodenum, and jejunum sections were examined using the FluoView software for image analysis (Olympus). Excitation, and barrier filters were set for rhodamin. The laser power and photomultiplier tube voltage were constant so that the fluorescence intensities of various samples could be compared. Images were digitized under constant gain and laser offset, with no post-capture modifications. Five section areas of the epithelium that contained the largest and brightest immunofluorescence for each sample were selected for measurement. The areas to be assessed were defined manually and used to normalize each peak intensity. The calculated mean fluorescence intensity was obtained for each of the selected section areas according to (Di Giancamillo et al, 2009). Pixel intensity was determined using the histogram/area functions of the FluoView software, which assigned the gray levels (GL) within a 0–256 Gy scale. Data were presented as mean fluorescence intensity.

Cecal microbiota evaluation: sample collection and DNA extraction.

At the slaughterhouse, cecal content samples were collected in sterile vials from 14 piglets (7 from CTR and 7 from T groups) and stored at −80 °C until DNA extraction. DNA was extracted from each sample using the QIAmp Fecal Pro kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and then it was stored at −20 °C until use.

16S Ribosomal RNA (rRNA) Gene Sequencing and Bioinformatics processing

Bacterial DNA was amplified using the primers described by Caporaso et al. (2011) which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 µL volumes per sample. A total of 12.5 µL of KAPA HIFI Master Mix 2× (Kapa 344 Biosystems, Inc., MA, USA) and 0.2 µL of each primer (100 µm) were added to 2 µL of genomic DNA (5 ng/µL). Blank controls (no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific). Samples were denatured at 95 °C for 3 min, followed by 25 cycles with a denaturing step at 98 ◦C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, 351 USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2×250-base paired-end reads.

The 16S rRNA gene sequences determined in this study were deposited in the NCBI Sequence Read Archive (SRA) database.

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC (Andrews, 2010). Reads were then cleaned by removing primers and adapters with the python tool Cutadapt (Martin, 2011), and by trimming for quality using the C++ tool Sickle (Joshi and Fass, 2011), with Phred threshold > 20 (i.e. the end part of the reads was removed if its quality deteriorated). After cleaning, forward and reverse paired-end reads were joined together using the python pipeline Micca (Microbial Community Analysis) (Albanese et al, 2015), specifically the function 'mergepairs' with default values (i.e. minimum overlap length = 32, maximum number of mismatches in the overlap region = 8). Assembled reads were filtered for quality, discarding reads with missing/uncalled bases or with an expected error rate larger than 1% (1 error in 100 bases). All remaining reads were used to identify OTUs (Operational Taxonomic Units) with the denoising approach (Rosen et al, 2012) implemented in the Micca function 'out' (method 'denovo_unoise'). Finally, the identified OTUs were classified using the MICCA function 'classify' to assign taxa as annotated in the SILVA132 reference database (Glöckner et al, 2017) with the following parameters: maximum number of hits -taxa- to

consider for each OTU= 3; assign taxon if present in at least 0.5 of the hits; reject OTU if the fraction of alignment to the reference sequence is lower than 0.75).

Statistical evaluation

A One-way ANOVA was applied to analyze TPC, FRAP, and ABTS through *in vitro* digestion phases. All experimental data relative to growth performance, fecal score, salivary IgAs, TAC, and cortisol were analysed as a completely randomized block design by ANOVA using the MIXED procedure of SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA) accounting for the effect of treatment, time, and their interaction. The model included the treatment, time, their interaction and the room. A general linearized model (GLM) was considered to evaluate duodenal and jejunal histometry and tight junction expression. When the data regarding tight junction expression were normally distributed, a One-Way ANOVA was performed, otherwise, the Kruskal-Wallis test was applied. These data were analyzed with GraphPad Prism 9.0.0 and are presented as means ± S.E.M. Pen represented the experimental unit for overall growth performances and fecal score. Individual piglet was considered as the experimental unit for single animal mean BW and ADG, as in the case of intestinal histometry and tight junction. Post-hoc evaluation was performed with a Bonferroni test. The OTU table obtained from 16S rRNA-gene sequencing was first filtered to remove OTUs with less than 10 total counts distributed in fewer than 3 samples. Filtered OTU counts were then normalised for uneven sequencing depth by cumulative sum scaling (CSS; Paulson et al, 2013). The normalised OTU table was used to calculate the alpha (ACE, Chao1, Fisher's alpha, Shannon, Simpson, Inverse Simpson) and beta (Bray-Curtis distances) diversity in the piglets gut samples. Details on the calculation of the alpha- and beta-diversity indices can be found in Biscarini et al. 2018 (Appendix S2). Differences between experimental groups (treatment and control) for OTU counts and alpha diversity indices were evaluated using analysis of variance. Differences between distance matrices were evaluated non-parametrically using the permutational analysis of variance approach (PERMANOVA with 999 permutations) (Anderson, 2001). Results were considered statistically significant for $P<0.05$ and highly significant for $P<0.01$. Graphs were realized with GraphPad Prism 9.0.0.

Results

Health status and adverse events

During the first two weeks of the study, 6 CTR group piglets and 1 T group piglet were treated with 1 ml Enrofloxacin (100 mg/ml) as they had diarrheal disorders. In addition, seven piglets were excluded from the test for the presence of abdominal hernias (3 CTR and 4 T). All the treated / excluded piglets were moved to infirmary pens.

In vitro digestibility: TPC, FRAP and ABTS assay

DM digestibility revealed a value of 49.68 ± 2.12 %. Moreover, for TPC, FRAP and ABTS followed a similar trend through *in vitro* digestion process. Briefly, in oral, and gastric phase TPC values were higher than intestinal phase (22193.68 ± 1301.74 mg TAE/100g and 25032.63 ± 1419.93 mg TAE/100g vs 4534.74 ± 549.13 mg TAE/100g; P<0.01, Fig. 1A). Similarly, oral, and gastric FRAP revealed the same trend in comparison with the intestinal phase (14048.32 ± 268.62 mg $FeSO_4$/100g and 15718.55 ± 297.06 mg $FeSO_4$/100g vs 4727.07 ± 167.91 mg $FeSO_4$/100g; P<0.01, Fig. 1B). ABTS values in oral and gastric phase were equally higher than intestinal one (88824.92 ± 6032.04 mg TE/100g and 92081.07 ± 5104.48 mg TE/100g vs 18820.56 ± 894.27 mg TE/100g; P<0.01, Fig. 1C).

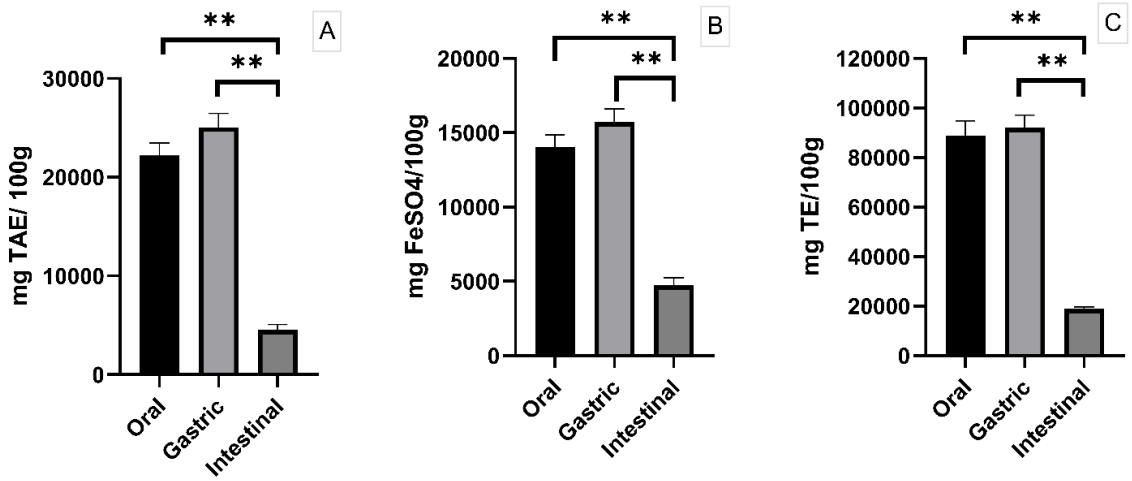

Figure 1. Total phenolic content (TPC), FRAP and ABTS results registered at the end of each digestion phase (A, B and C respectively). Values are expressed as mean ± standard error mean (S.E.M). * = P < 0.05, ** = P < 0.01

Fecal score

On days 6, 7 and 8 on trial, the treated group registered a better fecal consistency as depicted by the interaction between time and treatment variables ($P<0.05$, Fig. 2). Furthermore, the administration of the tested blend was useful in ameliorating fecal score of treated animals even when considering the treatment as single variable ($P<0.05$). Specifically, a difference between CTR and T was detected already at 6 d after weaning (1.00 ± 0.36 vs 0.71 ± 0.48, $P<0.05$, Fig. 2). Moreover, at 7 d CTR vs T comparison revealed a better fecal score for the treated group (1.71 ± 0.38 vs 1.14 ± 0.47, $P<0.05$, Fig. 2). Finally, this difference was also underlined at 8 d on trial comparing CTR vs T group (2.00 ± 0.46 vs 1.57 ± 0.37, $P<0.05$, Fig. 2).

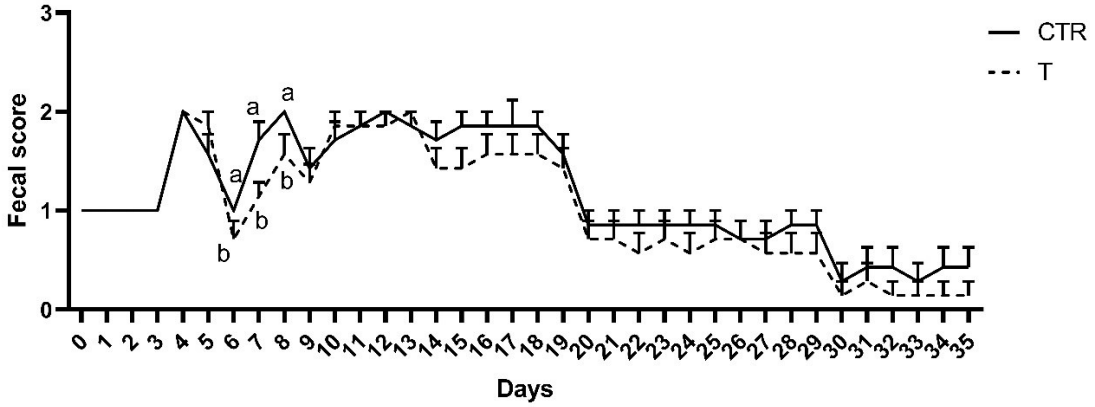

Figure 2. Fecal score evaluation performed during the trial (0-35 d) suggested a better consistency of feces of the T group compared to the CTR one from day 6 to 8 on trial. Values are presented as means ± standard error mean (S.E.M); different letters mean statistically significant results (a,b $P<0.05$). CTR: control group; T: treated group

Growth performances

The growth performances of individual piglets are shown in table 2 (individual weight and ADG), while the results related to pens as experimental units are shown in table 3. Despite the lack of significance in the interaction between treatment and time, the treated group fed with Carvacrol, TA, and MCFAs registered better growth performances during the trial. BW of T piglets was found to be higher compared to CTR (16.67 ± 3.13 kg vs 15.82 ± 2.79, $P=0.041$, table 2). Average daily gain (ADG) also improved in the T group (0.25 ± 0.087 kg vs 0.23 ± 0.078, $P=0.003$, table 2). On the other hand, data related to pens revealed a tendency towards better FCR and FE for the T group if compared to CTR (table 3).

Table 2. Growth performance of single post-weaning piglets after the administration of Carvacrol, TA, and MCFAs blend. All the values are intended as means ± standard error mean (S.E.M); BW=body weight; ADG=average daily gain. CTR: control group; T: treated group. Treatment, time and their interaction *p*-values are displayed in the *p*-value section of the table.

	Treatments			*p*-value		
Parameters	CTR	T	S.E.M	Treatment	Time	Time* Treatment
BW (kg)						
0 d	7.65	7.74	0.20	0.041	<.0001	0.144
14 d	9.03	9.23				
35 d	15.82	16.67				
ADG (kg/d)						
0 d	0.10	0.11	0.01	0.03	<.0001	0.259
14 d	0.32	0.35				
35 d	0.23	0.25				

Table 3. Growth performance of pens evaluated from day 0 to 35 on trial. All the values are intended as means ± standard error mean (S.E.M); BW=body weight; ADG=average daily gain; ADFI=average daily feed intake; FCR=feed conversion rate; FE=feed efficiency. CTR: control group; T: treated group. Treatment, time and their interaction *p*-values are displayed in the *p*-value section of the table.

Treatments				*p*-value		
Parameters	CTR	T	S.E.M	Treatment	Time	Time*Treatment
BW (kg)						
0 d	111.52	111.62	9.81	0.574	<.0001	0.797
14 d	131.64	133.17				
35 d	228.28	240.54				
ADG (kg/d)						
0-14 d	1.44	1.54	0.23	0.111	<.0001	0.670
14-35 d	4.60	5.11				
0-35 d	3.34	3.68				
ADFI (kg/d)						
0-14 d	2.71	2.81	0.19	0.351	<.0001	0.965
14-35 d	7.67	7.88				
0-35 d	5.69	5.85				
FCR						
0-14 d	1.95	1.86	0.08	0.091	0.005	0.953
14-35 d	0.60	0.65				
0-35 d	0.58	0.63				
FE						
0-14 d	0.53	0.54	0.02	0.087	0.003	0.828
14-35 d	0.60	0.65				
0-35 d	0.58	0.63				

Salivary cortisol, IgAs, and total antioxidant capacity (TAC)

At day 35d, a significative decrease in salivary cortisol was highlighted in the T group (0.672 ± 0.16 ng/ml vs 1.589 ± 1.03 ng/ml, P<0.05, Fig. 3A). No differences were detected in salivary IgA levels (Fig. 3B) and total antioxidant capacity between CTR and T group during the trial (Fig. 3C).

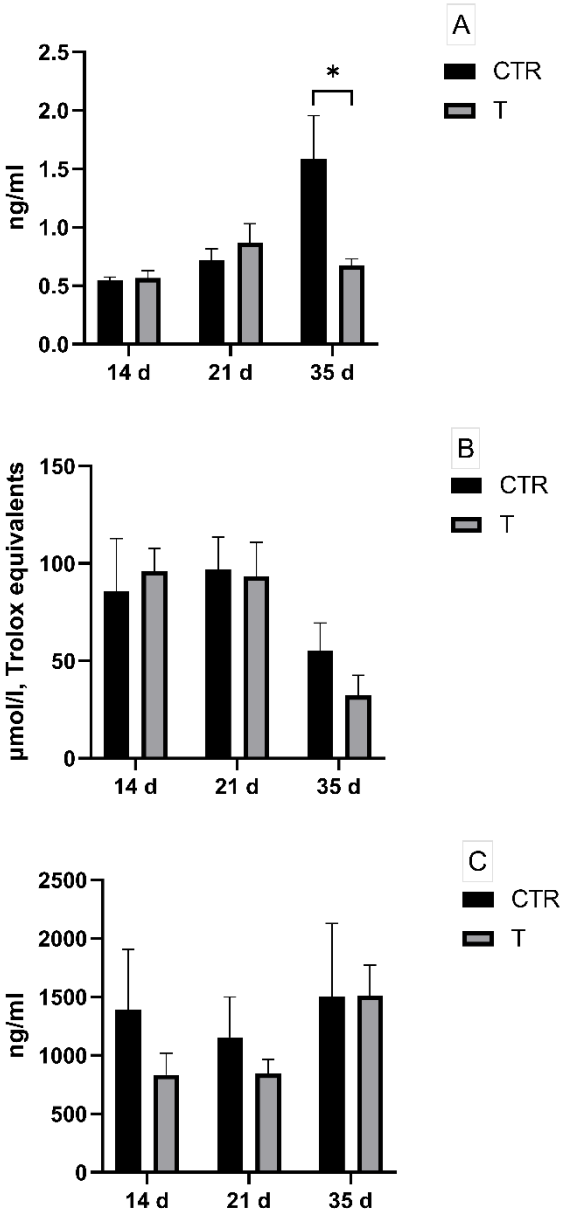

Figure 3. Salivary cortisol, TAC measured by FRAP and IgA (A, B and C respectively) registered at 14, 21, and 35 d on the trial in control (CTR) and treated (T) groups; n=7 per group. Values are expressed as mean ± standard error mean (S.E.M). * = P < 0.05, ** = P < 0.01

Intestine histology and histometry

Gut morphology of both control and treated animals was revealed to be structurally normal, with no sign of inflammation or epithelial detachment. Moreover, duodenal morphology was affected by the inclusion of the tested blend in the piglet's diet. A higher villi height was registered in the T group when compared with CTR (320.27 ± 68.53 µm vs 292.33 ± 90.35 µm; P<0.05, Fig. 4A). On the other hand, deeper crypts were evident in CTR samples (171.38 ± 50.56 µm vs 236.45 ± 90.38 µm; P<0.01, Fig. 4B). Consequently, the V/C ratio highlighted higher values for T samples when comparing them to CTR (2.16 ± 1.04 vs 1.37 ± 0.56; P<0.01, Fig. 4C). Jejunum revealed comparable results when analyzing the morphometric characteristics. Indeed, samples of the T group showed increased villi height (339.94 ± 107.76 µm vs 300.66 ± 101.74 µm; P<0.05, Fig. 4D), decreased crypts depth (144.83 ± 53.40 µm vs 208.82 ± 73.00 µm; P<0.01, Fig. 4E) and a higher V/C ratio (2.61 ± 1.21 µm vs 1.55 ± 0.65 µm; P<0.01, Fig. 4F).

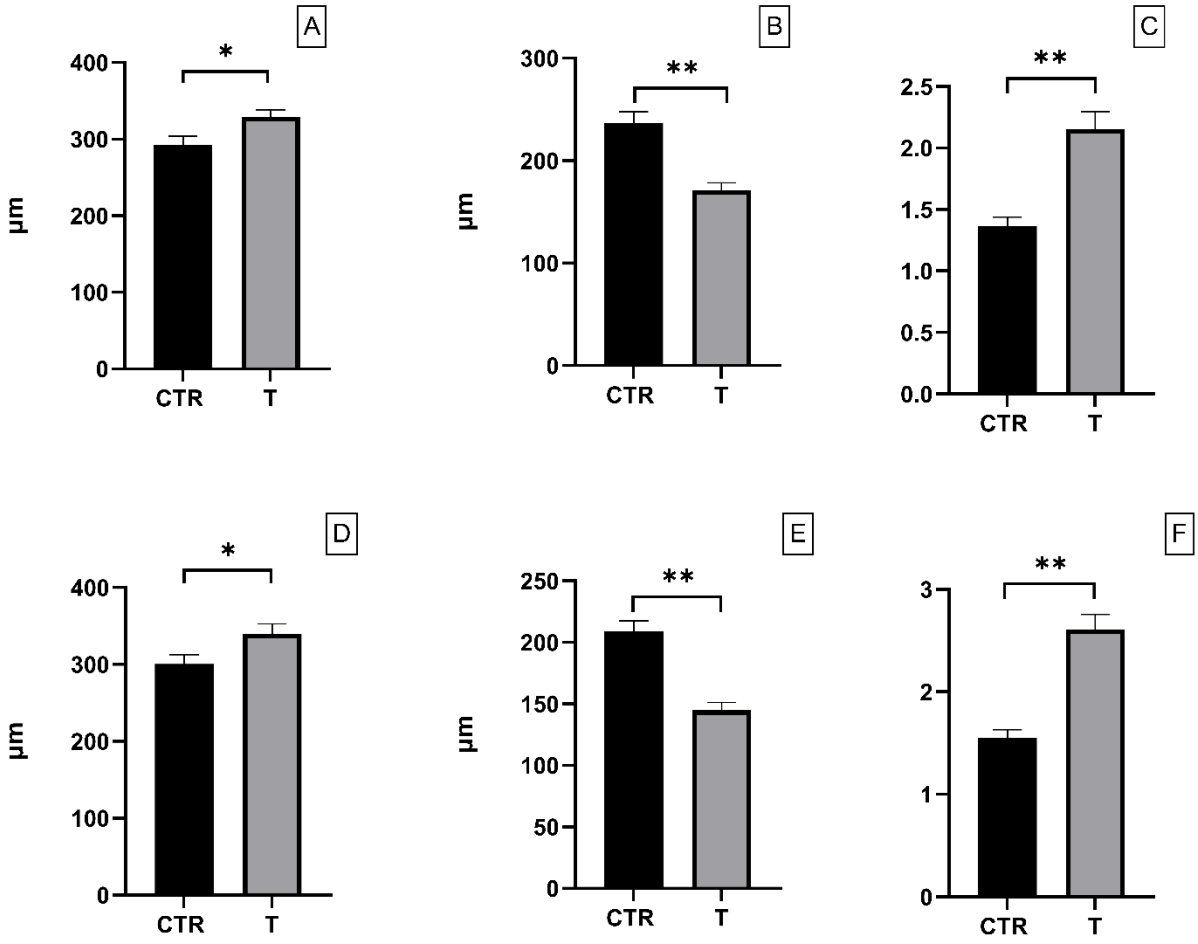

Figure 4. Villi height, crypt depth and V/C ratio registered in duodenum (A, B and C respectively) and Jejunum (D, E and, F respectively) at 35d in control (CTR) and treated (T) groups. Values are expressed as mean ± standard error mean (S.E.M). * = P < 0.05, ** = P < 0.01

Gut barrier assessment: E-Cadherin, Zonulin-1, and Occludin immunofluorescence staining

Junction proteins were specifically assessed along the length of the villi in both duodenum and jejunum of treated animals. E-Cadherin was mostly found in cell-cell contact junctions in both intestinal tracts (Fig. 5 A, duodenum and D, jejunum, green colour, arrows), throughout the length of the villi. Distinctly, ZO-1 was located only at the apical end of the enterocytes in both the duodenum and the jejunum (Fig. 5, B and E respectively, red color, bold arrows). Finally, Occludin was observed at apical and basolateral plasma membrane domains, either in the duodenum or the jejunum (Fig. 5 C and F, respectively, yellow colour, asterisks).

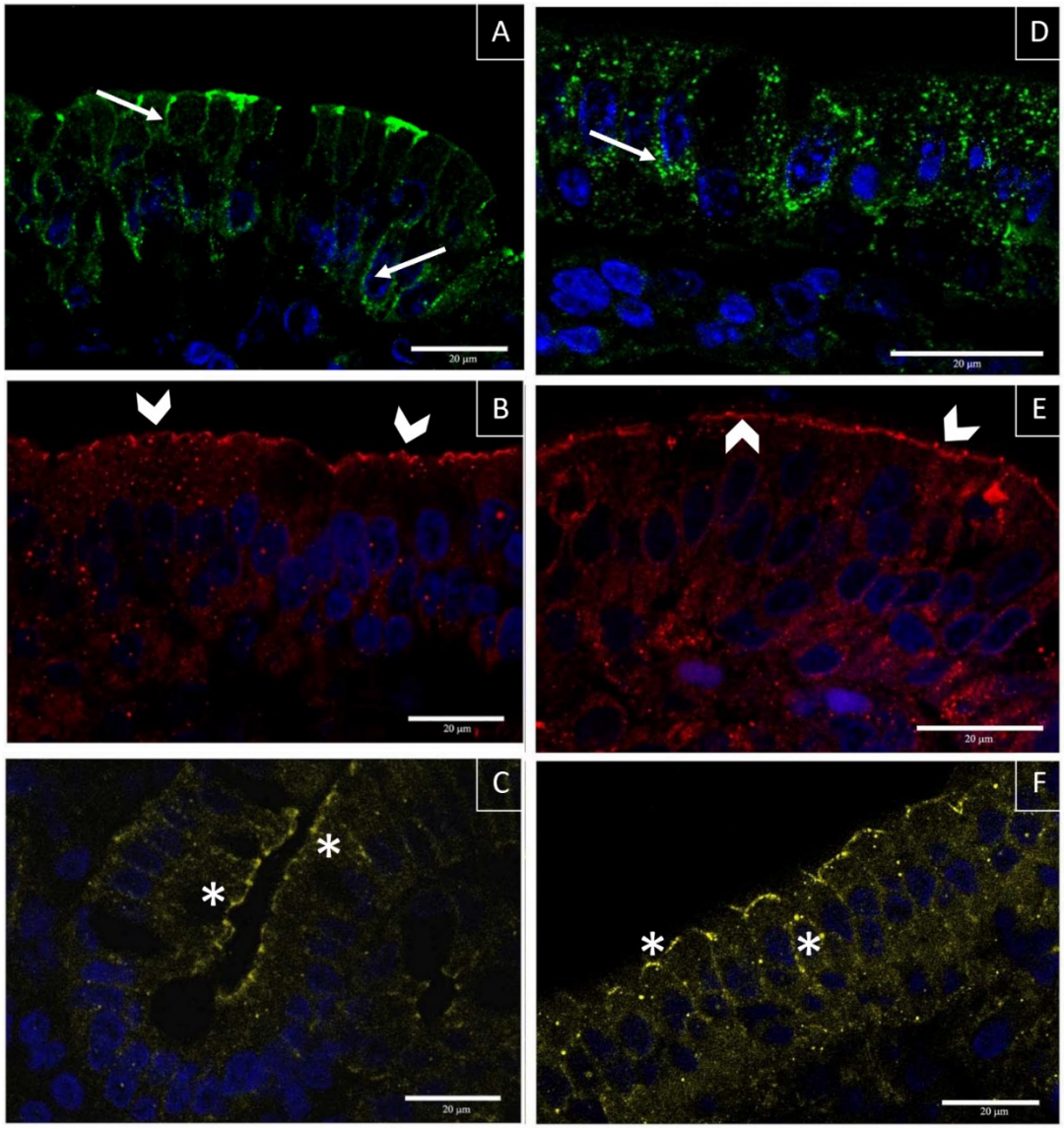

Figure 5. Representative images of immunofluorescence (IF) of E-Cadherin (A, D), Zonulin-1 (B, E), and Occludin (C, F) in treated animals in the duodenum (A, B, C) and in the jejunum (D, E, F). E-Cadherin IF in green, indicated by arrows; ZO-1 IF in red, indicated by bold arrows; Occludin IF in yellow, indicated by asterisks; nuclei, blue. Scale bar located in each image: 20 µm

Junction protein expression was also quantified (Fig. 6). At the duodenum level, statistical differences were found only with Occludin, where the control animals showed a higher expression (Fig. 6 C, $P< 0.05$). Regarding the jejunum, statistical differences were found for all three staining. Animals of the treated group showed higher expressions of E-Cadherin and Occludin (Fig. 6 D and F respectively, $P<0.05$), while with Zonulin-1, the result was the opposite (Fig. 6 E, $P < 0.01$).

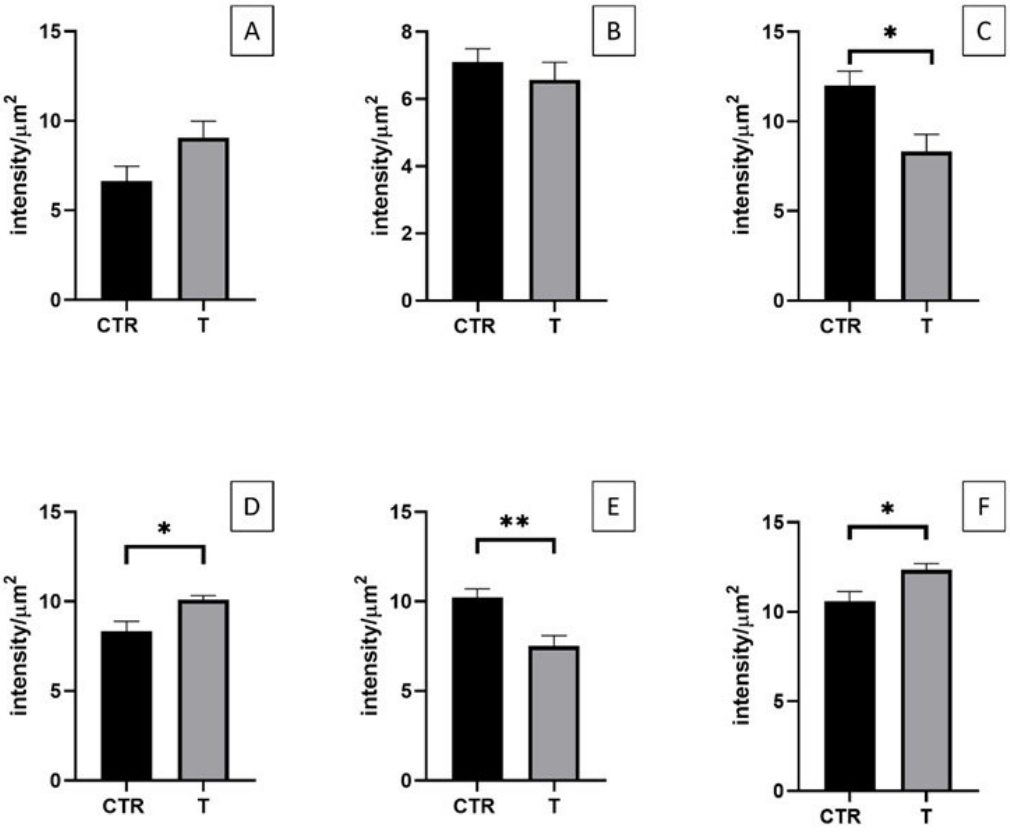

Figure 6. Quantitative representation of the expression of E-Cadherin in the duodenum (A) and in the jejunum (D), of Zonuline-1 in the duodenum (B) and in the jejunum (E), and of Occludin in the duodenum (C) and in the jejunum (F) of control (CTR) and treated (T) groups sampled at 35 d.Values are expressed in intensity per μm^2. One-way ANOVA was performed. Results are expressed as mean ± standard error mean (S.E.M). * = $P < 0.05$, ** = $P < 0.01$

Cecal Microbiota

The gut microbial diversity was assessed within- (alpha diversity) and between- (beta diversity) samples (Fig. 7). All indexes for alpha were estimated from the complete OTU, filtered for OTUs with more than 10 total counts distributed in at least three samples and normalized for uneven sequencing depth by cumulative sum scaling (CSS). Within-sample microbial richness and diversity were estimated using the following indices: Chao1 and ACE (Abundance-based coverage Estimator) for richness and, on the other hand, Shannon, Simpson for evenness and Fisher's alpha for diversity. Beta diversity was estimated based on Bray-Curtis distances (Fig. 8). None of the differences between treated and control samples for the diversity indices was significant (all p-values > 0.05). From PERMANOVA, the p-value for the treatment effect was 0.093. Therefore, both alpha and beta diversity of the piglets' gut microbiota were not significantly affected by the treatment.

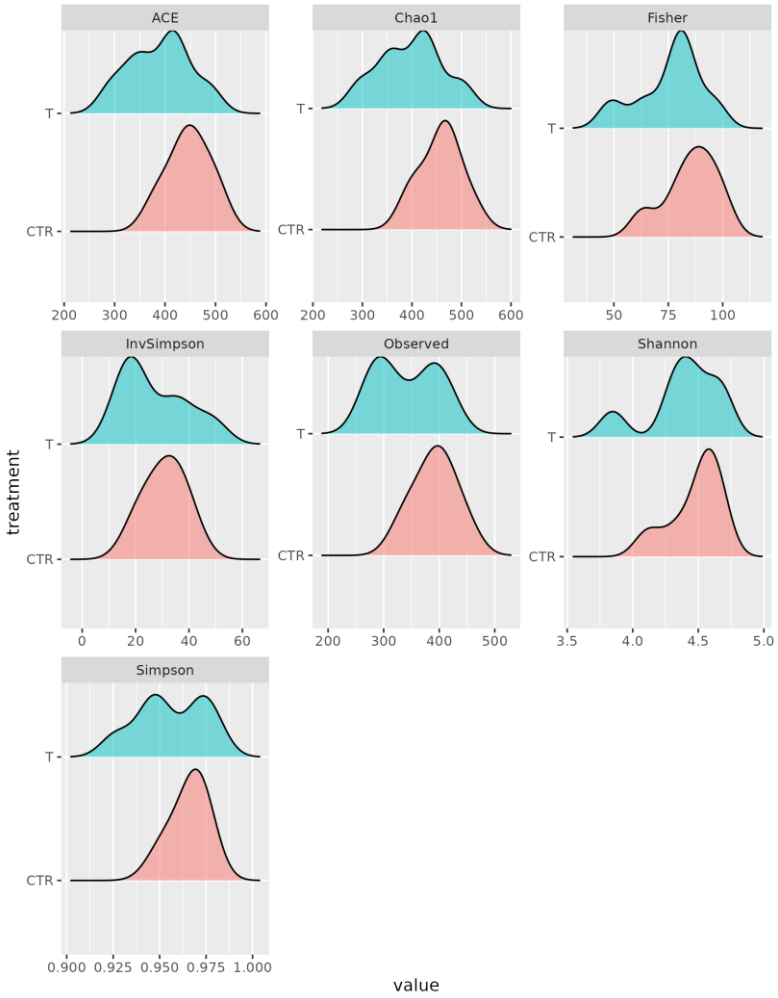

Figure 7. Density graph referred to alpha diversity evaluation performed on cecal samples of control (CTR) and treated (T) groups collected at the end of the trial (35 d)

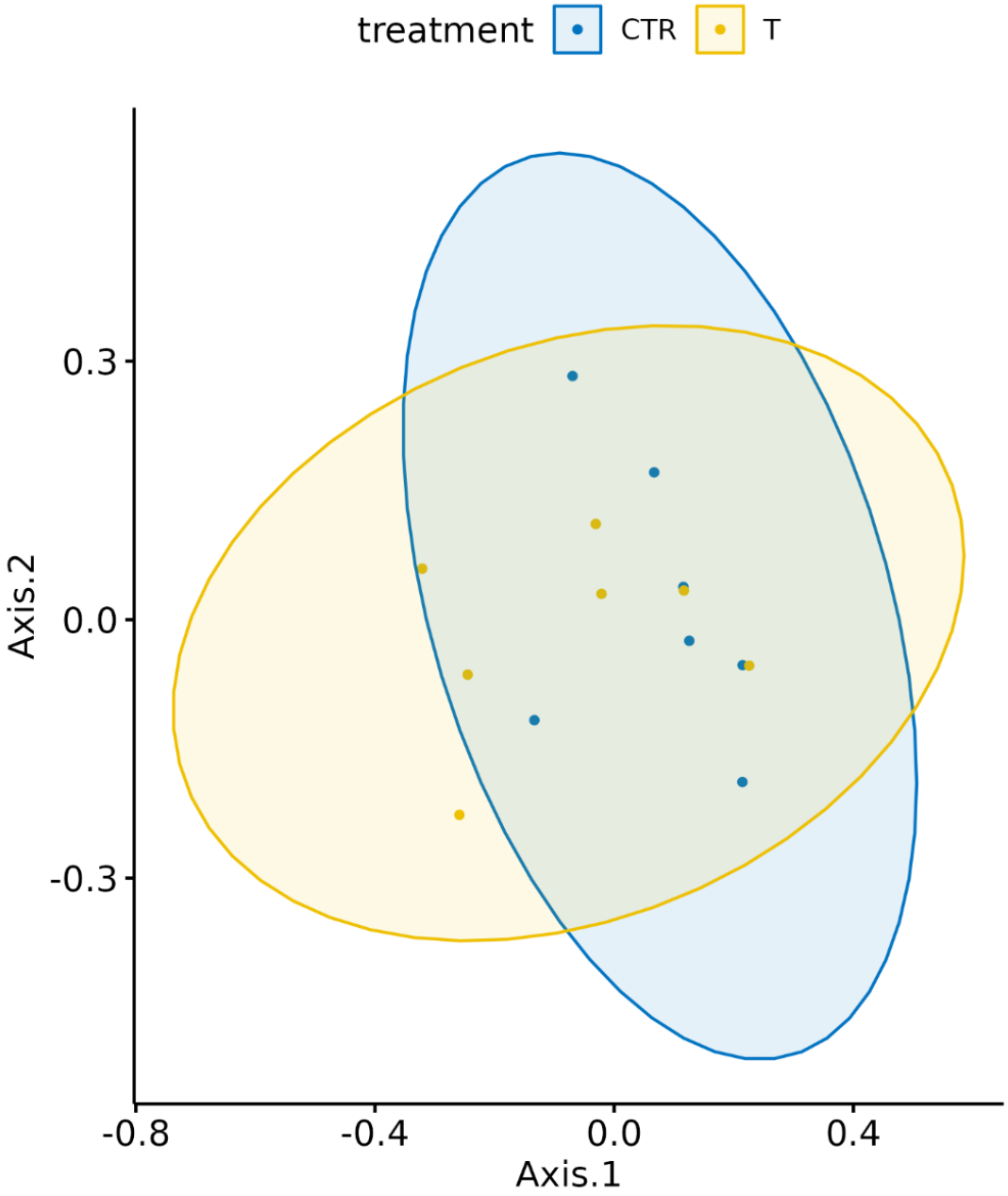

Figure 8. Ellipse graph referred to Bray-Curtis dissimilarities of control (CTR) and treated (T) groups evaluated on cecal samples collected at the end of the trial (35 d).

The 16S rRNA gene sequencing results from all cecal samples were used to define the core microbiota of piglets. OTUs were taxonomically organized in phyla and genera. Sequencing the V3-V4 regions of the bacterial 16S rRNA gene produced a total of 2,872,968 reads after filtering for quality. The resulting OTU table contained 729 OTUs that reduced to 409 OTUs after filtering for abundance and distribution. Phyla with relative abundance lower than 0.1% were not considered. A total of 12 abundant genera were significantly affected by the dietary treatment. In particular, the administration of the blend affected genera such as *Lachnospiraceae UCG-001*, *Lachnospiraceae UCG-008*, *Prevotellaceae NK3B31* group, *Clostridium sensu stricto 13* and *Ruminococcaceae UCG-005* (P<0.05). Phyla and genera average counts are displayed in figure 9 (A and B respectively).

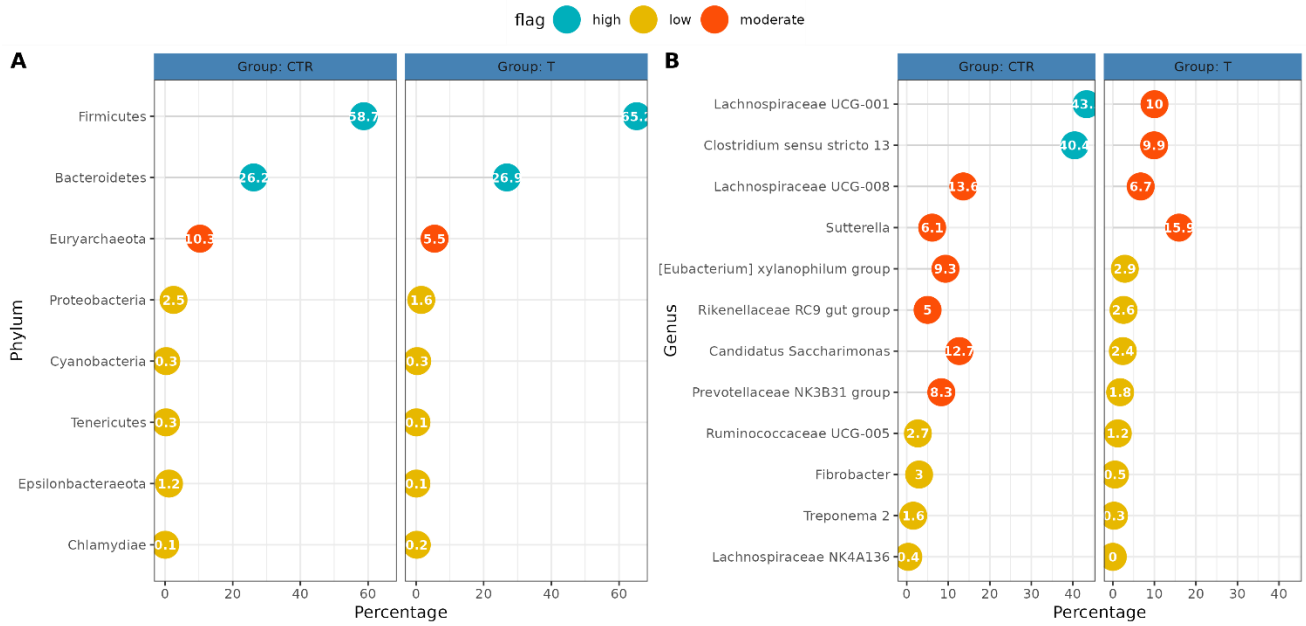

Figure 9. Percentage of average counts of phyla (A) and genera (B) detected in the two experimental groups. None of the differences between treatments for the reported phyla were significant (P>0.05). On the other hand, 12 abundant genera were affected by dietary treatment (P<0.05). CTR: control group; T: treated group.

Discussion

The blend considered in this study is composed of carvacrol, tannic acid (TA) from *castanea sativa* mill, *Glycyrrhiza glabra* extract, diglycerides and triglycerides of medium-chain fatty acids (C:8, C:10, and C:12; MCFAs). Carvacrol is a monoterpene phenol originally found in different varieties of *Origanum*, *Thymus,* and *Satureja* plants (Suntres et al, 2015). Carvacrol supplementation in animal feed is a valid strategy to promote gut health due to its antioxidant, anti-inflammatory, and antimicrobial properties (Alagawany et al, 2015). MCFAs are molecules characterized by the presence of 6 to 12 carbon atoms (Ferronato and Prandini 2020). Lauric, capric, and caprylic acid are naturally present in palm and coconut oil (Jadhav and Annapure 2023). As reported by Rebucci et al, (2021) to avoid the complete absorption of their free forms at the gastric level, MCFAS are administered as saponified or glycerides to promote gut health in the enteric tract without being directly dissociated in the gastric tract. Tannic acid (TA) is a naturally occurring and hydrolysable polyphenolic compound, characterized by the presence of heterogeneous phenol groups available to different molecular interactions (Watrelot et al, 2020). As in the case of MCFAs, TA bactericidal activity seems to be more effective towards gram-positive bacteria (Baldwin and Booth 2022). Blended feed additives recently gained an increasing interest in the feeding of post-weaning piglets. Moreover, the study of blends in livestock nutrition could be crucial to optimize the effectiveness of these molecules under varying conditions (Abdelli et al, 2021). Furthermore, blends characterized by different compositions may display positive effects on the intestinal environment of weanling piglets, contributing to preventing the reduction of antibiotics and representing an alternative to pharmacological dosages of ZnO (Luise et al, 2023). An *in vitro* characterization of the depicted blend anticipated the *in vivo* phase of the present study. Briefly, an overlapping trend was depicted when looking at TPC and antioxidant capacity over the digestion process (oral, gastric, and intestinal). Interestingly, the trend highlighted a peak after gastric digestion in TPC, FRAP, and ABTS assays which was followed by a marked decrease after the intestinal phase. The marked increase of TPC after the gastric phase might be due to the efficacy of acidic pH in breaking the polysaccharide bonds and the subsequently release of phenol compounds which are associated with antioxidant capacity as depicted by Lanzoni et al, (2023). As reported by the author, the significant decrease observed after the intestinal phase can be related to the instability of phenols in the intestinal environment (basic pH and/or pancreatic enzymes effect). Nevertheless, the antioxidant activity registered at the end of the digestion together with the high MCFAs content in the tested blend (23%) could highlight the positive effects further evidenced during the *in vivo* trial.

The first effect of the dietary blend inclusion was registered on animals performance. Shao et al, (2023) found an increased ADG and ADFI during the first 4 weeks after weaning in piglets fed a blend of essential oils containing 2.34% of Carvacrol. Moreover, Cui et al, (2020) registered better performances when considering different glycerides forms and combinations of lauric, capric, and caprylic MCFAs. Furthermore, despite the potential detrimental effect of tannins on performances due to limited protein digestibility, contained TA supplementation (1.13, 2.25, and 4.25 g/kg) enhanced feed efficiency during

the weaning transition (Biagia et al, 2010). In our study, the blend of Carvacrol, TA, and MCFAs improved the ADG and BW of weaned piglets, confirming the beneficial potential of these molecules reported in the literature. Although the effect of MCFAs and carvacrol has not been reported to constantly ameliorate the fecal consistency of post-weaning piglets alone or in combination with natural extracts (López-Colom P et al, 2020), it has been previously reported how TA and commercial tannins in general are useful tools to increase fecal score (Girard et al, 2018). Given that, the fecal score registered suggested a positive interaction of the different components in increasing the fecal consistency in critical stages of the post-weaning phase.

Saliva has been depicted to have great potential in displaying stress and disease characterization through the detection of stress, innate and adaptive immune response, and oxidative stress biomarkers in pigs (Sánchez et al, 2022). Moreover, salivary secretory immunoglobulin A is considered a natural defence for the host (Pedersen et al, 2019). In our study, no significant differences were found between CTR and T group in salivary IgAs. As reported by Muneta et al, (2010), Salivary IgAs could be influenced both by the circadian rhythm and feeding. In addition, Svobodová et al, (2014) indicated that the role of salivary IgAs was not confirmed when considering the short or long-term stress stimuli associated to weaning. Switching attention to salivary total antioxidant capacity (TAC), Saco et al, (2023) recently reported how this non-invasive biomarker could be strongly influenced by the production phase in addition to the circadian rhythm. According to Sánchez et al, (2022), there is a strong relationship between adaptive immune and oxidative stress salivary biomarkers. Perhaps, this could explain the absence of variations in the salivary TAC values registered in our trial when comparing the experimental groups. Given that, considering the lack of consistent pieces of evidence regarding the application of salivary IgAs and TAC in weanling piglets' nutritional studies, it can be stated that the variability of these two salivary biomarkers needs further investigation. Salivary cortisol has been confirmed as a reliable biomarker capable of describing the physiological conditions of pigs during critical phases of the production cycle (Bahnsen et al, 2021). Zhang et al, (2023) found that 50 mg/kg of a mixture of carvacrol, thymol, and cinnamaldehyde in a 1:1:1 proportion complex did not reduce cortisol levels in weanling piglets' blood. Furthermore, the lack of results in conditioning salivary cortisol levels through natural extracts and essential oils administration was previously underlined when considering different concentrations of garlic powder (0.4% and 1.2%) and oregano essential oil (0.4 % and 2.0 %), even though authors detected lower concentrations of serum cortisol of the piglets involved in the same study (Rivera-Gomis et al, 2020). On the contrary, in our study we detected a marked decrease of cortisol levels in saliva samples 35 d after weaning, suggesting a stimulation of the hypothalamic-pituitary-adrenal (HPA) axis, responsible for the glucocorticoid hormone production. Briefly, as depicted by Moeser et al, (2007) weaning stress is associated with the enhancement of the corticotropin-releasing factor (CRF) expression, which is linked to gut barrier dysfunctions. Due to this, it is reasonable to relate the physiological changes and stress that occur during the weaning transition to variations in cortisol levels (Wu et al, 2023) which served as indirect biomarker of gut barrier integrity. Therefore, our results

suggested a key role of blended carvacrol, TA, and MCFAs blend in lowering cortisol levels which served as indirect biomarker of gut health, as depicted also by further discussed results.

The ability of the tested blend in conditioning gut morphology was assessed through duodenal and jejunal histology and histometry. Mo et al, (2023) recently showed the capability of a 5 % carvacrol, 2 % thymol, and 3 % cinnamaldehyde blend to enhance the villus height and crypt depth ratio in the jejunum of weaned piglets. Furthermore, a blend of 1 g of both capric and caprylic acids modified the small intestine mucosal structure of weanling piglets decreasing crypt depth as depicted by Hanczakowska et al, (2011). On the other hand, Ferrara et al, (2016) underlined a lack of effects when considering the 0.15 % of both capric and caprylic acids blended with short-chain fatty acids in the jejunal morphometry. Moreover, when blending 10.1 % calcium formate 25.1 % of citric acid, and an essential oils mixture (4.5 % thymol, 4.8 % carvacrol and 4.3 % cinnamaldehyde) it is possible to increase villi height and influence V/C ratio as reported by Liu et al, (2022). Our results are in line with this picture, testifying an increased villus height and V/C ratio both in duodenum and jejunum, but also remarking a significantly enhanced crypt depth for the CTR group. Chwen et al, (2013) reported that deeper crypt depth is associated with a more intense turnover of the enterocytes, which could further result in higher villi. However, not always higher length villi are associated with an increased absorptive capacity. According to Pluske et al, (1997), the intestine may show lower absorptive capacity even with high villi length, when the enterocytes are not mature. It does not seem to be our case, considering the other positive results obtained in this study, such as body weight, fecal score, and saliva cortisol. Moreover, there are indications that, in germ-free animals, the increased villi height does not necessarily correspond to an increase in the absorptive capacity (Williams et al, 2015), but this is neither the case of our study. Thus, it is reasonable to speculate that the tested blend positively influenced the morphometric characteristics of the proximal intestine. Our results may be representative of a situation in which the treatment allowed a more rapid recovery of small intestine tissue conditions after weaning than in the control group.

TJ are complex structures comprising over 50 proteins and include a series of transmembrane proteins, such as Occludin and Zonulin. Adherens junctions, such as E-Cadherin, are located beneath the TJ and are involved in cell-cell adhesion and intracellular signaling, and all together these protein junctions regulate paracellular permeability (Krug and Fromm 2020). E-Cadherin is an adherents junction molecule, which plays a key regulatory role in barrier integrity through its temporal and spatial coordination of the tight junction (Itoh et al, 1997; Umeda et al, 2006). Additionally, Zonulin-1 is the major TJ protein and in normal intestines, it is expressed exclusively at the apical TJ (Fasano 2012). Furthermore, occludin is an integral membrane protein of epithelial tight junctions (Anderson and Van Itallie 2009; Wu et al, 2020).

Positive results were obtained by Zhao et al (2023) considering duodenal Occludin and ZO-1 mRNA expression after the administration of carvacrol–cinnamaldehyde–thymol blend. On the contrary, Wei

et al, (2017) found no effects when considering the supplementation of a blend of carvacrol and thymol essential oils at 100 mg/kg (1:1) on ZO-1 and Occludin expression in jejunal mucosa. In addition, a mixture of natural extracts was useful in enhancing the expression of E-Cadherin in the distal enteric tract of weaned piglets (Su et al, 2018). Briefly, Grilli et al, (2016) showed that sodium butyrate reduced the expression of claudin-1 in the duodenum and jejunum, while Occludin was regulated in the duodenum but not in the jejunum tract. Focusing on protein abundance, Zou et al, (2016) obtained positive results administering essential oils to weaned piglets when looking at the Occludin and ZO-1 expression in the jejunum tract. Moreover, Cui et al, (2020) registered an increased jejunal Occludin expression by administering 2 g/kg of glycerol monolaurate in weanling piglets. On the contrary, the author reported a lack of effects on ZO-1 and Occludin after two weeks of administration of tricaprylin and tricaprin mixture (1 g/kg each). Furthermore, a phytobiotic mixture derived from oregano extract composed of carvacrol and thymol showed no effects on ZO-1 and a significant decrease in Occludin levels in jejunum 21 d after weaning (Duarte and Kim 2022). The localization of the three molecules performed in our study is consistent with what is described in the literature, in particular, regarding E-Cadherin in cell-cell contact junctions throughout the length of the villi (Hwang et al, 2012; Xu et al, 2008; Zahn et al, 2008), ZO-1 at the apical end of the enterocytes (Dong et al, 2021; Fasano 2011; Kimura et al, 1997; Nouri et al, 2014; Aidos et al, 2023), and Occludin at apical and basolateral plasma membrane domains (Dong et al, 2021; Kimura et al, 1997). From the quantification of the protein expression, it seems that the jejunum is more susceptible to diet interventions, as differences in the expression of all TJs studied, E-Cadherin, Zonulin-1, and Occludin showed statistically significant differences in this tract. This is probably because the jejunum is the major site for nutrient absorption, while the duodenum is mostly dedicated to digestion (Campbell et al, 2019). However, in the duodenum, there was a difference in the expression of Occludin, which was opposite to the one observed at the jejunum level. Considering the duodenum and its digestive functions, it is reasonable to think that this result was conditioned by the presence of pancreatic enzymes and bile. Indeed, it is recognized how variations in pancreatic enzymes and bile salts can influence the bio accessibility of phytochemicals (Wojtunik-Kulesza et al, 2020). Considering the expression results of jejunal E-Cadherin and Occludin, it can be noticed that the treated animals showed a lower intestine permeability when compared with the control group animals, outlining a protective effect of the treatment on the intestinal barrier. However, the expression of ZO-1 showed an opposite trend: treated animals showed lower ZO-1 expression. The explanation for this results may be found in the role of Zonulin as an endogenous mediator in the physiological regulation of intercellular tight junctions in the small intestine (Fasano 2020, 2011; Fasano et al, 2000; Hałasa et al, 2017). Indeed, ZO-1 can reversibly modulate the permeability of the intestine (Fasano et al, 2020). For instance, an increase in the expression of zonulin with a subsequent increase in permeability, was observed in human intestinal diseases, like irritable bowel syndrome, non-celiac gluten sensitivity, environmental enteropathy, and necrotizing enterocolitis (Sturgeon et al, 2016).

Therefore, the higher expression of ZO-1 in the control group observed in this study may indicate an alteration in intestinal permeability in these animals.

Since abrupt weaning-related changes in the gut microbial core can negatively affect homeostasis, epithelia turnover and gut barrier functions with negative reflexes on general gut health (Ren et al, 2022; Blachier et al, 2017) cecal microbiota was evaluated in the presented study. Briefly, at genus level, modulation of *Ruminococcaceae*, *Rikenellaceae* and *Prevotellaceae* genera was registered. *Lachnospiraceae* has been previously linked to short chain fatty acids production, with possible positive reflexes on gut and host health (Jiangh et al, 2021). On the other hand, its role in piglets gut environment is still controversial and not clear (Wang et al, 2022). In addition, *Rikenellaceae* group displayed a potential role of intestinal health biomarker and was positively correlated with higher feed convertion rate (Quan et al, 2018). Briefly, *Prevotellaceae* NK3B31 and *Ruminococcaceae* UCG-005 have been link to the production of acetate and propionic acid derived from resistant starch degradation, as in the case of *Fibrobacter* which was linked to cellulose digestion in diverse hindgut fermenters (Wang et al, 2019; Gaukroger et al, 2020). Controversary findings were highlighted regarding the role of *Candidatus Saccharimonas*, which was found less abundant both in high and low feed efficiency pigs and high abundant in cecal samples of obese pigs in a metabolome-microbiota relation trial (Liu et al, 2023). Moreover, even without evidence about *Eubacterium xylanophilum* group presence and development in piglet gut during weaning, rumen isolates of *Eubacterium xylanophilum* were reported to degrade xylan and produce short chain fatty acids. Treponema 2 sequences have been linked to higher feed efficiency, lignin degradation and higher presence of *Methanobrevibacter* in large intestine of pigs (Gardiner et al, 2020). Regarding *Sutterella*, in humans this genus has been previously correlated to inflammatory bowel diseases conditions and IgA degradation (Shapiro et al, 2021). On the contrary, more recently *Sutterella* has been positively related to feed intake and lipids digestion in commercial hybrid pigs (Luo et al, 2022). *Sutterella* along with *Proteobacteria* was found to be particularly abundant in diarrheic Tibetan early weaned piglet by Kong et al, (2022). Furthermore, an increasing number of studies are relating *Clostridium sensu stricto* 13 to inflammatory enteric diseases and diarrhea (Wang et al, 2023; Chen et al, 2022). In our case, despite a reduced presence of *Ruminococcaceae*, *Lachnospiraceae* and *Prevotellaceae*, the treated group registered a lower abundance of *Clostridium sensu stricto* 13. Considering what was previously assessed, it is more probable that the direct effect of the blended compounds on duodenal and jejunal histometry and gut barrier integrity reflected a reduction of *Clostridium sensu stricto* 13 at 35 d rather than directly act on its abundance. Therefore, it is reasonable to link the reduction of *Clostridium sensu stricto* 13 to a better status of the intestinal barrier.

Conclusions

The administration of a blend composed of Carvacrol, tannic acid, and MCFAs improved the gut health of weaned piglets ameliorating fecal consistency, physiological stress status, gut tissue morphometry

and permeability, and reflecting changes in *Clostridium sensu stricto* 13 abundance. Nevertheless, differences in compositional terms of the compared blends must be considered. In conclusion, further research is needed to elucidate the synergistic effect and different inclusion levels of these substances when applied in commercial farm conditions.

Ethical statement

The experimental design and procedures were approved by the Animal Welfare Committee of Università degli Studi di Milano (OPBA 12_2022, 8/04/2022).

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Disclosure statement

The authors reported no potential conflict of interest.

Data availability statement

Upon reasonable request, data are available from the corresponding author: luca.marchetti1@unimi.it

Authors contributions

Conceptualization: Valentino Bontempo, Raffaella Rebucci, Luca Marchetti; Methodology: Raffaella Rebucci, Luca Marchetti, Davide Lanzoni, Carlotta Giromini, Lucia Aidos, Alessia Di Giancamillo, Paola Cremonesi; Formal analysis and investigation: Luca Marchetti, Raffaella Rebucci, Paola Cremonesi, Filippo Biscarini; Writing - original draft preparation: Luca Marchetti; Writing - review and editing: Luca Marchetti, Raffaella Rebucci, Davide Lanzoni, Carlotta Giromini, Alessia Di Giancamillo, Bianca Castiglioni; Supervision: Valentino Bontempo.

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CHAPTER 7: Dietary supplementation with potentiated zinc and monovalent copper oxide for weanling piglets: effects on systemic and mucosal immunity, gut permeability and microbiota composition

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Brief introduction to the study: this part of the project was carried out to evaluate the potential of a balanced administration of Zn and Cu supplied through more bioavailable sources in post-weaning piglets. After the ban on pharmacological ZnO in June 2022, the interest in elucidating the biological relationship between Zn and Cu increased. The idea behind this study was to optimize both trace elements effects on gut health testing different ratios and comparing them to a pharmacological administration of ZnO during piglets weaning. With this study I gained the possibility to participate in a first international conference at 74th European Federation of Animal Science (EAAP) meeting helded in Lyon (France, 26th August-1st September 2023) . This experience markedly contributed both to my personal and professional growth. The data from this study were elaborated and utilized to prepare a manuscript submitted in Porcine Health Management journal (BMC, Part of Springer Nature) which is under revision.

Abstract

The ban on pharmacological zinc oxide dosages intended for weanling piglets has enhanced the attention to the biological interplay between copper and zinc. However, the relationship between zinc and copper in conditioning gut health during the weaning transition needs further evaluations. The present study hypothesized the possibility to modulate piglets gut health parameters through different Zn/Cu ratios administered via specialty oxide sources. A total of 84 piglets were selected after weaning and divided into 4 experimental treatments and the trial lasted 28 d. During the first phase (0-14 d), a positive control (PC) fed 2500 ppm of zinc via conventional zinc oxide (ZnO) was accompanied by 3 treatments in which Cu and Zn were supplemented through potentiated ZnO (Pot-ZnO) and monovalent copper oxide (Cu_2O) at European and Non-European levels of inclusion: EU (120 ppm of Zn; 140 ppm of Cu), Non-EU^+ (300 ppm of Zn; 200 ppm of Cu) and Non-EU^- (300 ppm of Zn; 140 ppm of Cu). The second phase (14-28 d) was characterized by lower Zn/Cu ratios. Better fecal score was detected on day 4 in PC group when compared to EU ($P<0.01$). Serum IgA increased in Non-EU^- group vs PC ($P<0.05$) at 14 d, while serum diamine-oxidase as indirect marker of intestinal permeability was lower in PC and Non-EU^+ vs Non-EU^- ($P<0.05$). Jejunal SIgA increased in PC vs Non-EU^- ($P<0.01$) at 28 d. Zn was higher in PC fecal samples ($P<0.01$), whereas fecal Cu was conditioned by treatments ($P<0.05$) at 14 d. Analysis of fecal microbiota showed a decreased biodiversity in Non-EU^- and Non-EU^+ when compared to EU ($P<0.05$). Beta diversity highlighted a significant separation among groups at 14d ($P<0.01$). Differential abundances performed at genus level, revealed that fecal microbiota composition was conditioned by different Zn/Cu ratios at 14 d ($P<0.05$). Excess of Zn could manifest limitation in ameliorating gut health parameters due to probable enhanced Cu sequestration. Balanced Zn/Cu ratios administered via specialty oxide sources and within European levels of inclusion may represent a valid strategy to enhance gut health of piglets during the first two weeks after weaning.

Keywords: nutrition, trace elements, gut health, feed additives, weaning

Introduction

The weaning period represents one of the most critical phases in pig production in which the pressure of social, feeding, and physiological changes could favor the outburst of post weaning diarrhea (PWD). Furthermore, the establishment of the microbial gut core could be compromised by the presence of undigested nutrients in the intestine, having long-term reflexes on gut tissue because of the altered crosstalk activity between microbiota and gut mucosa (Tang et al, 2022). Thus, to avoid these complications, the administration of Zn and Cu in pharmacological dosages through zinc oxide (ZnO) and copper sulphate ($CuSO_4$) inorganic salts have been extensively studied in the past (Ma et al, 2015; Luise et al, 2024). Generally, trace elements are supplied in piglets diet through inorganic salts formulations, characterized by very low bioavailability and, consequently, potential detrimental effects on the environment (Hejna et al, 2018). Due to this, the European Community promoted the ban of pharmacological dosages of Zn and, previously, remodulated the maximum permitted level of inclusion of Cu in weanling piglets diet. In particular, Zn is allowed at a maximum level of 150 mg/kg in piglet feed during the weaning period, whereas Cu can be included up to 150 mg/kg of complete feed for the first 4 weeks after weaning, and up to 100 mg/kg from week 5 to week 8 (Commission Implementing Regulation (EU) 2018/1039). On the other hand, eastern countries still allow the administration of 1600 mg/kg of Zn during the first two weeks after weaning and 200 mg/kg of Cu (Sun et al, 2019; Lin et al, 2020).

As alternatives, more bioavailable sources of trace elements have been increasingly studied. Enhanced bioavailability is mainly based on an increased surface of trace elements particles, that could also give the possibility to gain positive results in terms of biological interactions through reduced trace elements concentrations (Cho et al, 2013).

After ingestion, ZnO and $CuSO_4$ dissociate into ions taking part in different metabolic pathways and pillar enzymatic reactions. Briefly, Zn^{2+} ions could acquire a direct catalytic function or structural role as stabilizing element of several enzymatic processes related to metalloenzymes (Thompson et al, 2022). On the other hand, copper ions are widely known for their role as redox transition metal elements able to participate in scavenging of free radicals, iron metabolism and neurological functions (Jomova et al, 2022). Furthermore, the affinity of these ions with metalloproteins such as metallothioneins could be the main factor responsible for the regulation of trace element homeostasis. Indeed, the presence of cysteine sequences in metallothioneins binds a number of trace elements (Dong et al, 2014). Dalto et al. (2023 a, b), recently reported how overlying Zn dosages and low concentrations of Cu could enhance metallothioneins expression having direct consequences on both trace elements metabolism in weaned piglets. Therefore, when administered at high dietary levels, Cu and Zn are barely absorbed in the intestine, affecting the availability of other nutrients (Nishito et al, 2018).

Commonly, while ZnO is associated with changes in the gram-positive fraction of the intestinal microflora, Cu is widely known for its capacity to affect gram-negative bacteria (Højberg et al, 2005).

In addition, copper has a well-known capacity of modulating the gut microbiota, by decreasing coliforms, and *Streptococcus spp.* (Villagómez-Estrada et al, 2020). Moreover, several studies demonstrated that ZnO could also decrease the counts of Lactobacilli and Bifidobacteria in gut environment. However, the containment of coliforms proliferation could be more related to the host local immunity response modulation and to direct improvement of the intestinal barrier conditions which remain still unclear (Subramanian et al, 2016).

Administering Zn and Cu to piglets through alternative formulations revealed positive effects in terms of bioavailability, gut health, and immunity. Porous and nano particles formulations of ZnO improved the expression of anti-inflammatory pathways related to cytokines and showed positive effects on performances, small intestine histometry and fecal score during the weaning transition (Long et al, 2019). Moreover, the inclusion of porous ZnO in weanling piglets diet showed a remarkable modulation of intestinal barrier tight junctions and systemic immunity (Peng et al, 2019). On the other hand, alternative formulations of Cu such as Nano Cu or tribasic copper chloride increased performances of weaned piglets, improving intestinal morphometry, and displaying a bactericidal effect towards gram negative pathogens with potential modulatory effect on gut microbiota composition (Kim et al, 2021; Lin et al, 2020). These positive results testified the suitability of alternative formulation in weanling piglets diet instead of classic ZnO and $CuSO_4$ inorganic salts forms.

Potentiated ZnO (Pot-ZnO) is an alternative processed form of ZnO characterized by a surface that is 10 to 15 times extended in comparison to the conventional form. Pot-ZnO is a porous zinc oxide formulation characterized by smaller particles, higher bioavailability, and a more diffused distribution on the tissue surface with recognized positive reflexes on gut health (Long et al, 2017); monovalent copper oxide formulation (Cu_2O) represents an alternative to the more common $CuSO_4$ and showed beneficial effects on growth performances of both piglets and broilers when administered in extra-nutritional dosages, with a lower copper accumulation in liver (Forouzandeh et al, 2022). Furthermore, alternative formulations of ZnO have been indicated as potential modulators of the intestinal microflora, modulating *Enterobacteriaceae and Lactobacillus spp.* (Oh et al, 2022; Zhang et al, 2022).

To the best of our knowledge, there is a lack of studies that investigated the reflexes on gut health of different Zn/Cu ratios administered through alternative sources. In addition, given the dose-dependent effect of these molecules, it is clear from the literature that many of the results obtained have been highlighted through Non-European levels of inclusion of both trace elements, precisely above 150 mg/kg of complete feed. Therefore, we decided to investigate the effects of Pot-ZnO and Cu_2O administered different ratios based both on European and Non-European levels of inclusion of Zn and Cu comparing them to a pharmacological administration of conventional ZnO during the weaning transition.

Material and methods

Experimental design and animal housing

Immediately after weaning corresponding to 28 d of life and 0 d of trial, a total of 84 crossbreed Topigs piglets, half male and half females (7.143 ± 0.924 kg) were selected from the farm "Azienda Agricola Arioli-Sangalli" (Genzone, Pavia, Italy) and transported to the experimental facilities of the Department of Veterinary Medicine and Animal Sciences at the University of Milan, Italy. Piglets were allocated according to body weight to 4 experimental treatments through a randomization process to guarantee that each treatment consisted of an equal number of homogeneous replicates. Piglets were placed in 28 replicates organized as follow: 7 replicates per treatment with 3 subjects for each pen. Animals were allocated in pen with plastic grating flooring with 1.2 m^2 of free surface. The trial lasted 28 d. Animals were raised in a single room with controlled environmental conditions. The temperature was settled at 28 °C at 0 d and regulated weekly until reaching 24 °C at the end of the trial. Relative humidity was maintained below 65% and the airflow has been set at 10 m^3/animal/h. Water and feed were available *ad libitum* from day 0 on trial. Animals were fed a unique basal diet formulated to satisfy the nutrient requirements suggested by NRC (2012) for weanling piglets (Tab.2). Piglet were organized in 4 groups (Tab. 1): positive control (PC), European levels of inclusion of Zn and Cu (EU), Non-European levels of inclusion of Zn and Cu (Non-EU^+) and Non-European levels of inclusion of Zn group (Non-EU^-). PC group was fed the basal diet supplied with ZnO (72% of Zn) and $CuSO_4$ (25% of Cu) formulations while the remaining groups were fed potentiated zinc oxide (Pot-ZnO, HiZox® 75% of Zn) and monovalent copper oxide (Cu_2O, CoRouge®, 75% of Cu). As depicted by table 1, the experiment was divided into two different phases: phase 1 (0-14 d) in which higher dosages of Zn and Cu were considered, while phase 2 (15-28 d) was characterized by the administration of lower Zn/Cu ratios. The cited compounds were provided by Animine, 10 Rue Léon Rey Grange, 74960 Annecy, France.

Table 1. Experimental Design of the trial.

Treatment	Phase 1 0-14 d	Phase 2 15-28 d
PC	2500 ppm Zn (ZnO)	150 ppm Zn (ZnO) 150 ppm of Cu ($CuSO_4$)
EU	120 ppm Zn (Pot-ZnO) 140 ppm Cu (Cu_2O)	120 ppm Zn (Pot-ZnO) 140 ppm Cu (Cu_2O)
Non-EU$^+$	300 ppm Zn (Pot-ZnO) 200 ppm Cu (Cu_2O)	150 ppm Zn (Pot-ZnO) 200 ppm Cu (Cu_2O)
Non-EU$^-$	300 ppm Zn (Pot-ZnO) 140 ppm Cu (Cu_2O)	150 ppm Zn (Pot-ZnO) 140 ppm Cu (Cu_2O)

PC: positive control group; EU: European levels of inclusion of Zn and Cu group; Non-EU$^+$: Non-European levels of inclusion of Zn and Cu group; Non-EU$^-$: Non-European levels of inclusion of Zn group.

Feed was mixed in 100 kg batches for each treatment. To guarantee an adequate and homogeneous mixing process, the calculated amount of the different treatment compounds necessary for 100 kg of basal diet was weighted on a microbalance and premixed with a total of 8 kg of feed in a MLH laboratory mixer (WAMGROUP S.p.A.). Then, the premix was added to the remaining 92 kilograms of basal diet and remixed for a total time of 5 minutes. All the procedures were executed before distributing the feed to animals. Before each batch preparation the mixer was properly cleaned to avoid cross contamination among treatments. Furthermore, feeds samples were analysed for crude protein, crude fibre, ether extract, and ash (AOAC, 1997). Cu and Zn concentration in feed were determined by an ICP emission spectrometer (OPTIMA 3300 XL, Perkin-Elmer Corp., Waltham, MA, USA as described in Xue et al., (2014). Results of feed analysis are showed in table 3 and 4 and presented as mean ± standard deviation.

Growth performance and fecal score evaluation

Regarding growth performance evaluation, pen was considered as experimental unit. Piglets were weighted on day 0, 14 and 28 on trial to assess the body weight (BW) and the average daily gain (ADG). Contextually, removable feed trays were weighted, to calculate the average daily feed intake (ADFI), the feed conversion ratio (FCR) and the feed efficiency (FE). Fecal score was evaluated daily through a Bristol stool scale from 0 (normal) to 7 (severe diarrhea) as suggested by Longstreth et al. (2006). Piglets were checked daily by the veterinarian responsible for animal care.

Blood sampling, interleukins, immunoglobulins, permeability markers and antioxidant status assessment

On day 0, one subject per replicate was selected based on the average body weight of the pen for blood sampling. Therefore, on day 0 and 14, blood sampling was performed collecting 5 ml of blood directly from the jugular vein through a Vacutainer test red tube coated with microscopic silica particles (10 ml) armed with a 20G needle (VACUETTE®, Greiner Bio-One GmbH). Serum aliquots were obtained through a centrifugation at 3000 rpm for 15 minutes and then stored at −20 °C until analysis. Pro-inflammatory cytokines (IL-1β and IL-6) and immunoglobulins (IgA, IgG, and IgM) were analyzed using enzyme-linked immunosorbent assays specific for swine (Immunological sciences, Società Italiana Chimici, Rome, IT). Gut permeability markers (Diamino oxidase and L-lactate) and serum total antioxidant capacity (T-AOC) were analyzed through colorimetric assays (Immunological sciences, Società Italiana Chimici, Rome, IT).

Jejunal alkaline phosphatase and SIgAs quantification

At the end of the trial, animals previously selected for blood sampling were slaughtered to collect jejunal mucosa. Small intestine was removed, and the jejunum was quickly isolated, flushed with ice-cold PBS, scraped by slide to obtain mucosa samples, snap frozen in liquid nitrogen and stored at −80 °C. Jejunal mucosa 100 mg aliquots were prepared in 2 ml microtube (Sarstedt AG & Co) and homogenized in 1 mL of PBS. SIgAs and alkaline phosphatase were then quantified using ELISA kit tests (Immunological sciences, Società Italiana Chimici, Rome, IT).

Zinc and copper quantification.

Cecal and fecal trace elements concentration were determined following the methodology depicted by Zhuo et al. (2019). Briefly, 50 mg of sample were added to a Teflon tube prepared with 8 ml of concentrated nitric acid. A 20 min microwave digestion at 180 °C was applied. After cooling the system, samples were diluted with deionized water. Finally, Zn and Cu concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (OPTIMA 3300 XL, Perkin-Elmer Corp., Waltham, MA, USA).

Feces and cecal content sampling and sequencing

Fecal samples were collected at 14 and 28 d in sterile vials from 28 piglets (7 PC, 7 EU, 7 Non-EU^{+} and, 7 non-EU^{-}); cecal samples were collected from the same piglets at the end of the trial after slaughtering (28 d). Samples were stored at −80 °C until DNA extraction. DNA was extracted from each sample using the QIAmp Fecal Pro kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Extracted DNA was stored at −20 °C until use.

16S Ribosomal RNA (rRNA) Gene sequencing

Bacterial DNA was amplified using the primers described by Caporaso et al. (2011) which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 µL volumes per sample. A total of 12.5 µL of KAPA HIFI Master Mix 2× (Kapa 344 Biosystems, Inc., MA, USA) and 0.2 µL of each primer (100 µm) were added to 2 µL of genomic DNA (5 ng/µL). Blank controls (no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific). Samples were denatured at 95 °C for 3 min, followed by 25 cycles with a denaturing step at 98 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, 351 USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2×250-base paired-end reads.

Bioinformatics processing

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC (Andrews, 2010). Reads were then cleaned by removing primers and adapters with the python tool Cutadapt (Martin 2011), and by trimming for quality using the C++ tool Sickle (Joshi & Fass 2011), with Phred threshold > 20 (i.e. the end part of the reads was removed if of low quality). After cleaning, forward and reverse paired-end reads were joined together using the python pipeline Micca (Microbial Community Analysis; Albanese et al. 2015), specifically the function ‘mergepairs’ with default values (i.e. minimum overlap length = 32, maximum number of mismatches in the overlap region = 8). Assembled reads were filtered for quality, discarding reads with missing/uncalled bases or with an expected error rate larger than 1% (1 error in 100 bases). All remaining reads were used to identify OTUs (Operational Taxonomic Units) with the denoising approach (Rosen et al. 2012) implemented in the Micca function ‘otu’ (method ‘denovo_unoise’). Finally, the identified OTUs were classified using the MICCA function ‘classify’ to assign taxa as annotated in the SILVA132 reference database (Glöckner et al. 2017) with the following parameters: maximum number of hits -taxa- to consider for each OTU= 3; assign taxon if present in at least 0.5 of the hits; reject OTU if the fraction of alignment to the reference sequence is lower than 0.75). The obtained OTU table was filtered by removing least represented OTUs with < 20 counts in less than 4 samples. OTU counts were normalised for uneven sequencing depth by cumulative sum scaling (CSS; Paulson et al. 2013).

Alpha and beta diversity

Cecal and fecal microbial diversities were assessed within-samples (alpha diversity) and between-samples (beta diversity). All indexes for alpha were estimated from the complete OTU, filtered for OTUs with more than 10 total counts distributed in at least two samples and normalized for uneven sequencing depth by cumulative sum scaling (CSS). Within-sample microbial richness and diversity were estimated using the following indices: Chao1 and ACE (Abundance-based coverage Estimator) for richness and, on the other hand, Shannon, Simpson for evenness and Fisher's alpha for diversity. The across-samples microbiota diversity was assessed through Bray-Curtis dissimilarities. Among groups and pairwise Bray-Curtis dissimilarities were determined Non-parametrically using the permutational analysis of variance approach (999 permutations).

Table 2. Basal diet administered to animals from 0 to 28 d.

Ingredients (% as fed)	
Wheat meal	18.48
Extruded wheat	17.00
Barley meal	15.80
Bakery by-products	9.00
Dehulled flacked barley	8.80
Extruded soybean	6.90
Sweet whey	5.28
Soybean meal 48%	4.00
Flacked maize	4.00
Herring meal	2.60
Soy protein Concentrate 52%	1.50
Soybean hulls	1.40
Animal fat	1.00
L-Lysine	0.74
Soybean oil	0.60
Calcium formate	0.50
Dicalcium phosphate	0.37
L-Threonine	0.335
DL-Methionine	0.214
Sodium chloride	0.200
Calcium sulphate	0.190
L-tryptophan	0.091
Vitamins and trace elements premix[1]	1.00
Calculated nutrients values (% as fed)	
DM, %	88.89
CP, %	16.70
EE, %	5.34
CF, %	3.00
Zn, mg/kg	15.00
Cu, mg/kg	6.00
NE, kcal/kg	2.473

DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fibre; NE: net energy.

[1]Supplements (per kg as fed): Vitamin A: 10,000 IU; Vitamin D3: 1,000 IU; Vitamin E: 50 mg; Vitamin B1:1.0 mg; VitamineB2: 3.0 mg; Vitamin B12: 0.02 mg; Vitamin B6: 3,0 mg; Pantothenic acid: 10 mg; Nicotinic acid :15 mg; Biotin: 0.06 mg; Vitamin PP: 0,35 mg; Folic acid: 0,99 mg; Vitamin K3: 2 mg; Choline: 300 mg; Fe: 100 mg; Co:0.75 mg; Mn: 10 mg; I: 0.75 mg; Se: 0.4 mg.

Statistical analysis

Consistency of feces, and growth performances were analyzed by the GLM procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Serum concentrations of pro-inflammatory cytokines, immunoglobulins and serum antioxidant capacity, DAO and L-lactate were analyzed through a GLM procedure of SAS. Data collected on day 0 were considered as covariates for all the previously indicated parameters. Cecal and fecal Zn and Cu content, jejunal sIgAs and alkaline phosphatase were evaluated with a GLM procedure of SAS Treatment comparisons were done using Bonferroni significant difference test for multiple testing. Pens were considered as experimental unit for growth performances and fecal score evaluation, while single animals were considered when analyzing serum, gut mucosa, fecal and cecal samples. Results were considered statistically significant for $P<0.05$ and highly significant for $P<0.01$.

The comparison of alpha diversity and OTU abundances between experimental groups was evaluated using the following linear model:

$$y_{ijk} = \mu + \beta_1 * timepoint_j + \beta_2 * treatment_k + e_{ijk} \quad (1)$$

where: y_{ijk} is the alpha diversity index value or OTU abundance for sample i from treatment k at timepoint j; timepoint is the effect of time (day 14 or day 28); treatment is the effect of the dietary treatment (PC, EU, non-EU+, non-EU-); e_{ijk} are the model residuals. For cecum samples, collected at only one timepoint (day 28), equation (1) was simplified by removing the unnecessary timepoint_j effect. Bray-Curtis dissimilarities among groups were evaluated non-parametrically using the permutational analysis of variance approach (999 permutations), partitioning the variance across timepoints (only for fecal samples) and treatments (for all samples). Results were considered statistically significant for $P<0.05$ and highly significant for $P<0.01$.

Table 3. Feed analysis performed on phase 1 feed samples (0-14 d).

Parameters (% as fed)	**PC**	**EU**	**Non-EU^{+}**	**Non-EU^{-}**
DM, %	88.78 ± 3.80	88.75 ± 3.80	88.79 ± 3.80	88.77 ± 3.80
CP, %	16.81 ± 0.79	16.55 ± 0.78	16.23 ± 0.76	16.43 ± 0.75
EE, %	5.71 ± 0.35	5.85 ± 0.36	5.61 ± 0.35	5.74 ± 0.35
CF, %	3.09 ± 0.20	3.18 ± 0.20	3.00 ± 0.20	3.04 ± 0.20
Ash, %	4.33 ± 0.28	3.97 ± 0.26	4.01 ± 0.26	3.94 ± 0.26
Cu, mg/kg	6.50 ± 1.60	135.00 ± 21.00	212.00 ± 33.00	129.00 ± 19.00
Zn, mg/kg	2405.00 ± 220.00	131.00 ± 26.00	303.00 ± 44.00	309.00 ± 42.00

Analyses were performed on samples collected after the mixing process (n=5 per treatment). DM: dry matter; CP: Crude protein ; EE: ether extract ; CF: crude fibre ;Cu: copper; Zn: zinc. Data are presented as mean ± standard deviation.

Table 4. Feed analysis performed on phase 2 feed samples (14-28 d)

Parameters (% as fed)	**PC**	**EU**	**Non-EU^{+}**	**Non-EU^{-}**
DM, %	88.77 ± 3.60	88.78 ± 3.80	88.80 ± 3.70	88.79 ± 3.80
CP, %	16.58 ± 0.74	16.32 ± 0.77	16.43 ± 0.71	16.62 ± 0.78
EE, %	5.68 ± 0.33	5.81 ± 0.38	5.45 ± 0.30	5.51 ± 0.37
CF, %	3.10 ± 0.20	3.19 ± 0.20	3.16 ± 0.20	3.15 ± 0.20
Ash, %	4.31 ± 0.26	4.02 ± 0.24	3.98 ± 0.24	3.96 ± 0.27
Cu, mg/kg	139.00 ± 22.00	128 ± 24.00	182 ± 34.00	143 ± 25.00
Zn mg/kg	132.00 ± 26.00	114 ± 21.00	141 ± 28.00	152 ± 23.00

Analyses were performed on samples collected after the mixing process (n=5 per treatment). DM: dry matter; CP: Crude protein ; EE: ether extract ; CF: crude fibre ;Cu: copper; Zn: zinc. Data are presented as mean ± standard deviation.

Results

Fecal score

Fecal score was registered daily. As reported in Fig. 1, the only significant difference was found on day 4 between PC and EU ($P<0.01$). In addition, no other differences were detected during the overall trial.

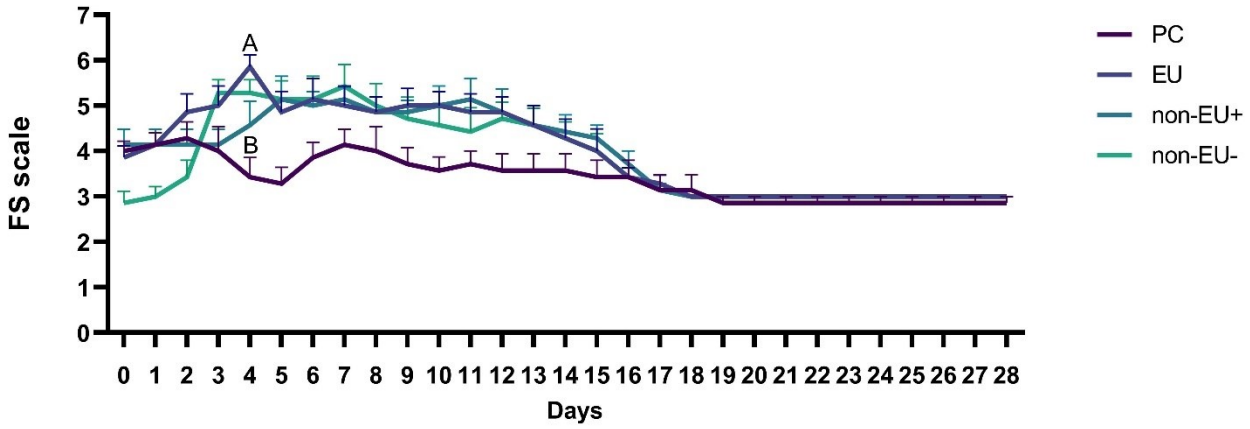

Figure 1. Fecal score registered daily during the trial through a fecal score scale (FS scale). n=7 per group. Different letters marks statistically significant results (A, B $P<0.01$). Data are presented as mean ± standard error mean (S.E.M).

Growth performances

As reported in Table 5, BW, ADG, ADFI, FCR and FE were not affected by the experimental treatments.

Table 5. Performances of pens registered during the trial (n=7 per group).

Parameters	PC	EU	Non-EU+	Non-EU-	S.E.M	P-value
BW (kg)						
0-14 d	22.60	22.55	22.61	22.60	0.89	1.00
14- 28 d	32.82	31.55	31.55	31.10	1.26	0.79
0-28 d	50.57	50.43	51.01	51.59	1.68	0.96
ADG (kg/d)						
0-14 d	0.730	0.643	0.638	0.607	0.04	0.25
14- 28 d	1.27	1.35	1.39	1.46	0.05	0.09
0-28 d	0.99	0.99	1.01	1.03	0.04	0.88
ADFI (kg/d)						
0-14 d	1.27	1.18	1.12	1.19	0.08	0.58
14- 28 d	2.14	2.28	2.23	2.34	0.13	0.74
0-28 d	1.71	1.73	1.67	1.77	0.09	0.91
FCR						
0-14 d	1.75	1.85	1.79	1.97	0.09	0.41
14- 28 d	1.67	1.68	1.61	1.60	0.06	0.66
0-28 d	1.70	1.63	1.65	1.70	0.05	0.76
FE						
0-14 d	0.58	0.55	0.58	0.51	0.03	0.36
14- 28 d	0.60	0.60	0.63	0.63	0.02	0.68
0-28 d	0.59	0.58	0.61	0.59	0.03	0.76

BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed convertion rate; FE: feed efficiency. Data are presented as mean ± standard error mean (S.E.M).

Pro-inflammatory cytokines, immunoglobulins and serum antioxidant capacity

Overall, pro-inflammatory cytokines IL-1β and IL-6 were not affected by the treatments during the trial (Fig. 2, A and B). Furthermore, the detected levels of serum IgG and IgM showed no significant differences among treatments (Fig. 3, A and B). As indicated by Fig. 3 (C) Non-EU$^-$ group recorded markedly higher levels of serum IgA in comparison with the CTR group (3723.78 ± 1086.52 ng/ml vs 1808.11 ± 450.78 ng/ml; P<0.05) on day 14 after weaning. Finally, serum antioxidant capacity was not conditioned by the treatments (Fig. 4, A and B).

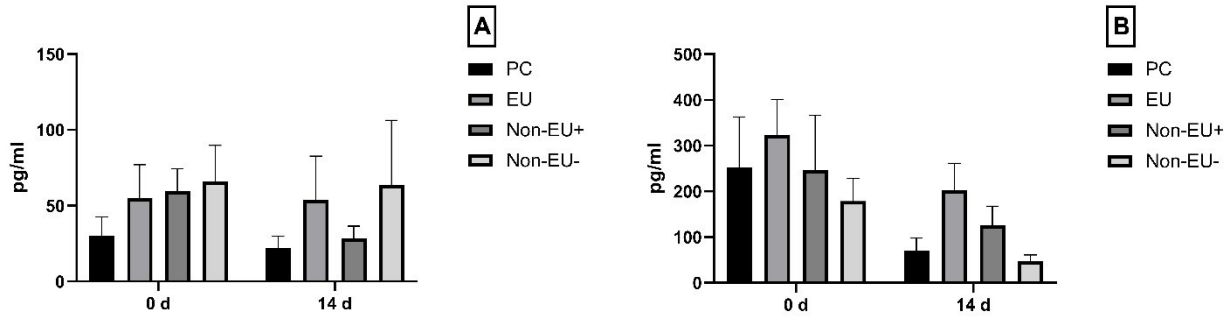

Figure 2. IL-6 (A) and IL-1β (B) analyzed at 0 and 14 d after weaning (n=7 per group). Data are presented as mean ± standard error mean (S.E.M).

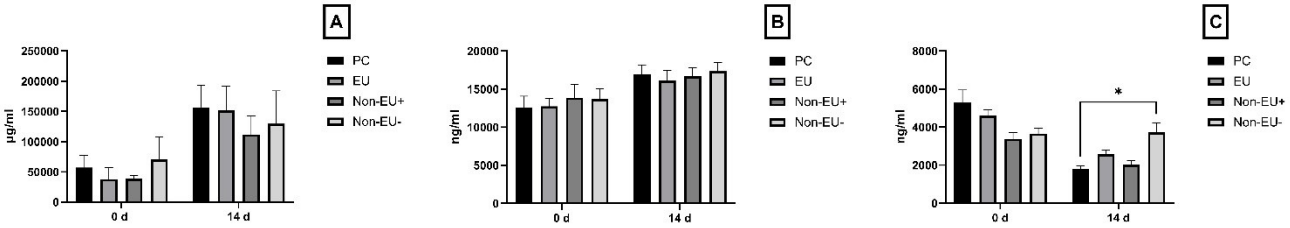

Figure 3. IgG (A), IgM (B) and IgA (C) quantification 0 and 14 d on trial (n=7 per group). Pairwise comparisons are evidenced as follows: *= P<0.05 **=P<0.01. Data are presented as mean ± standard error mean (S.E.M).

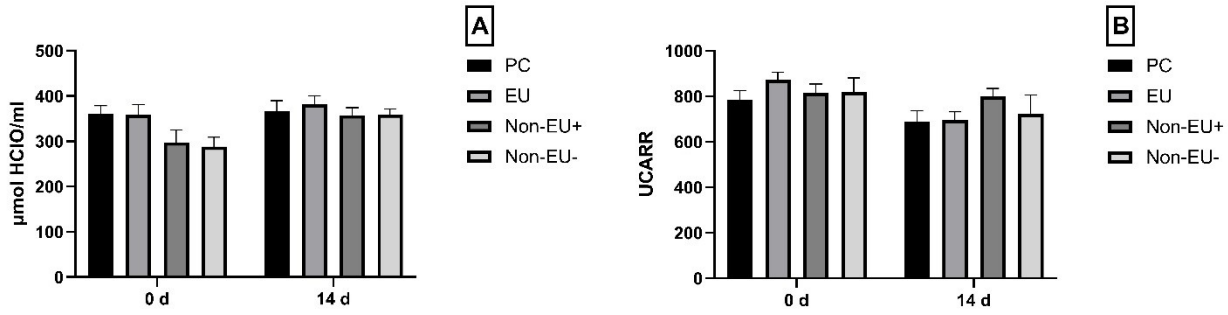

Figure 4. Serum oxidative status evaluated at 0 and 14 d (n=7 per group) through total antioxidant capacity (T-AOC, A) and D-ROMs (B) tests. Data are presented as mean ± standard error mean (S.E.M).

Intestinal permeability markers

No effects of treatments were detected on L-Lactate quantified in serum samples at 0 and 14 d after weaning (Fig. 5, A). On the contrary, DAO levels (Fig.5, B) were significantly conditioned by an unbalanced administration of Cu and Zn through alternative sources. Results showed a marked enhancement of DAO levels at 14 d in Non-EU^{-} group when compared with PC (91.16 ± 20.57 ng/ml vs 51.43 ± 21.58 ng/m; P<0.05) and Non-EU^{+} (91.16 ± 20.57 ng/ml vs 58.00 ± 31.69 ng/ml; P<0.05).

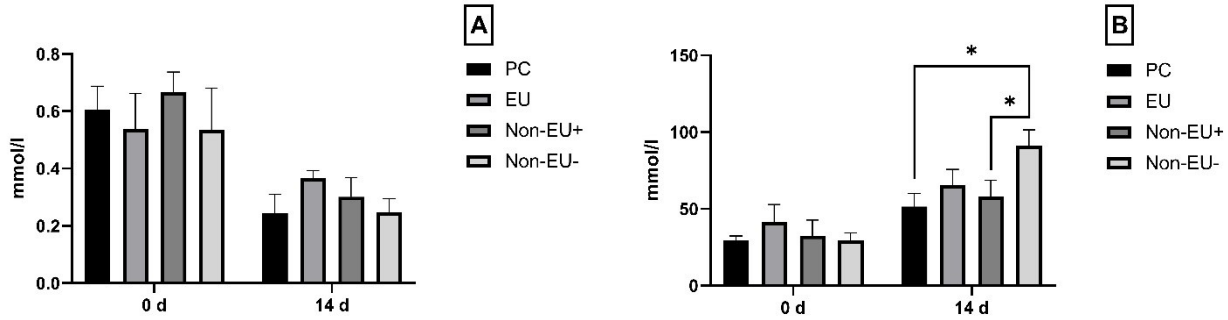

Figure 5. L-lactate and DAO determined in serum samples at 0 and 14 d on trial (A and B respectively, n=7 per group). Pairwise comparisons are evidenced as follows: *= P<0.05 **=P<0.01. Data are presented as mean ± standard error mean (S.E.M).

Zinc and copper content in fecal and cecal samples

Zinc levels in fecal samples collected at 14 d revealed that PC control group showed a higher Zn concentration when compared to EU, Non-EU^{+} and Non-EU^{-} (368.76 ± 42.11 mg/kg vs 30.04 ± 15.16 mg/kg, 43.53 ± 35.49 mg/kg and, 11.31 ± 6.24 mg/kg respectively; P<0.01, Fig. 6, A). As depicted in Fig. 6 (B) EU group showed higher levels of Cu at 14 d when compared to PC (25.00 ± 9.32 mg/kg vs 4.16 ± 0.46 mg/kg; P<0.05) and Non-EU^{-} (25.00 ± 9.32 mg/kg vs 6.69 ± 3.15 mg/kg; P<0.01, Fig. 6, B). Moreover, Non-EU^{+} evidenced higher copper concentrations in comparison with both PC and Non-EU^{-} (28.92 ± 23.28 mg kg vs 4.16 ± 0.46 mg/kg and 6.69 ± 3.15 mg/kg respectively; P<0.01). in addition, no effects were detected on trace elements concentration at 28 d after weaning. Neither Zn nor Cu cecal content determination revealed appreciable differences at the end of the trial (Fig. 7, A and B).

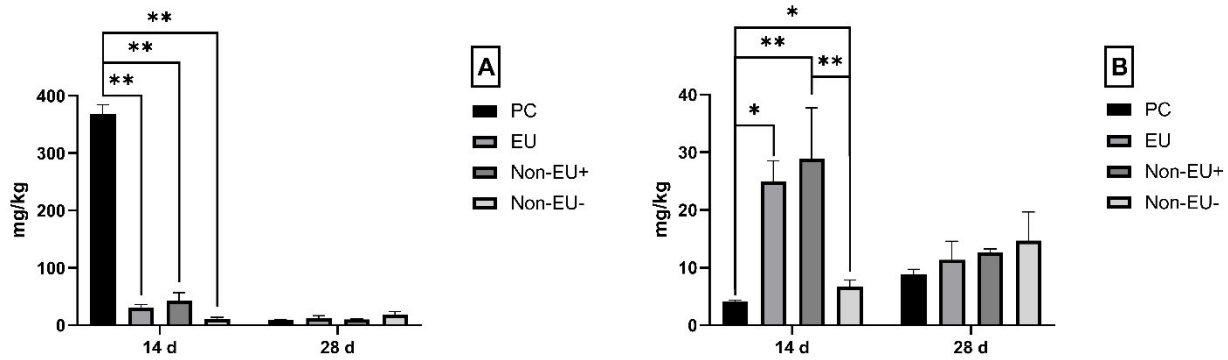

Figure 6. Zn and Cu levels determined in fecal sample collected at 14 and 28 d on trial (A and B respectively). Pairwise comparisons are evidenced as follows: *= P<0.05 **=P<0.01. Data are presented as mean ± standard error mean (S.E.M).

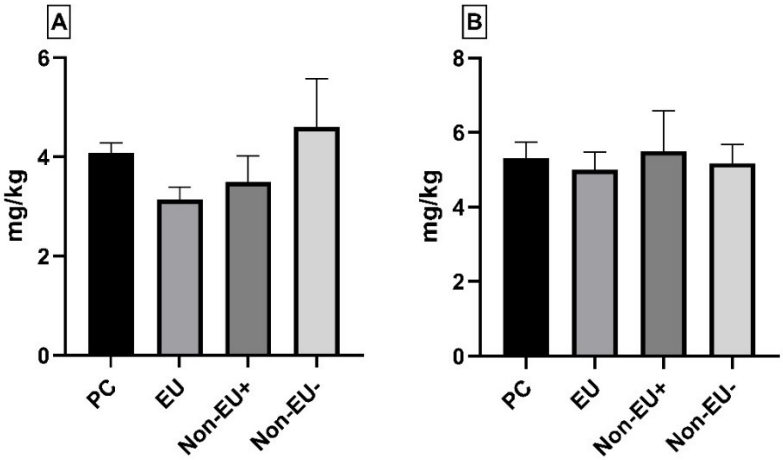

Figure 7. Zn and Cu levels detected in cecal content samples collected at 28 d (A and B respectively, n=7 per group). Data are presented as mean ± standard error mean (S.E.M).

Jejunal SIgAs and ALP

Jejunal SIgAs showed to be significantly conditioned by the administration of 2500 mg/kg of Zn through ZnO formulation at 28 d after weaning (Fig. 8, A). Briefly, PC group highlighted a higher level of SIgAs compared to Non-EU$^-$ group (157.73 ± 19.58 ng/ml vs 138.01 ± 16.88 ng/ml; P<0.05). Moreover, the jejunal mucosa alkaline phosphatase activity at 28 d was not conditioned by treatments (Fig. 8, B)

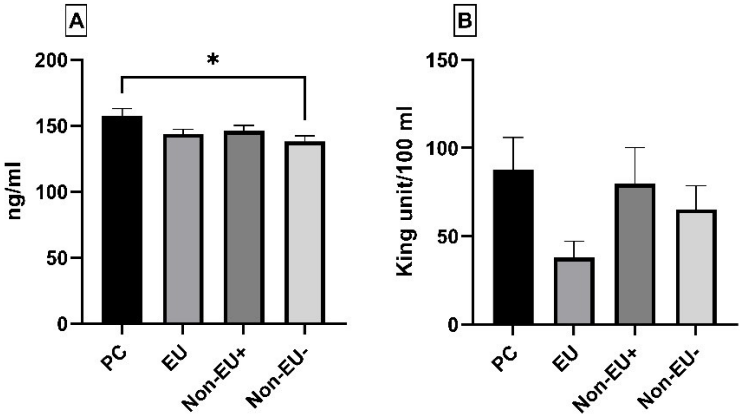

Figure 8. Jejunal level of SIgAs (A) and ALP (B) registered on day 28 after weaning (n=7 per group). Pairwise comparisons are evidenced as follows: *= P<0.05 **=P<0.01. Data are presented as mean ± standard error mean (S.E.M).

Cecal and fecal microbiota evaluation

Alpha and Beta diversity

Data regarding alpha diversity are presented in table 6. Fisher's alpha diversity index was reduced in PC group when looking at the contrast comparison with Non-EU$^-$ group in cecal samples collected at 28 d (P<0.05). EU fecal samples collected at 14 d showed higher values for Observed, Shannon and Simpson metrics in comparison with Non-EU$^-$ (P<0.05). On the other hand, EU displayed a higher Simpson metric value than Non-EU$^+$ (P<0.05). Briefly, fecal samples collected at 28 d revealed no effect of treatments in conditioning biodiversity.

Table 6. Average alpha diversity values (± standard deviation) for PC, EU, Non-EU+ and Non-EU- groups.

Sample	metric	timepoint	PC	EU	Non-EU+	Non-EU-
feces	ACE	D14	1808.8 ± 330.8	1974.8 ± 171.9	1744.7 ± 167.4	1620.1 ± 296.4
feces	Chao1	D14	1809 ± 337.9	1973.1 ± 172.4	1730 ± 170.5	1617.6 ± 299.3
feces	Fisher	D14	324.5 ± 48.2	347.3 ± 32.8	321.9 ± 32.3	301 ± 68.2
feces	InvSimpson	D14	67.7 ± 14.9	82.6 ± 20.9	52.6 ± 37.9	49.1 ± 26.9
feces	Observed	D14	1680.6 ± 315.6	1829.4 ± 165.1 **a**	1607.3 ± 168.3	1467.4 ± 279.1 **b**
feces	Shannon	D14	5.591 ± 0.067	5.657 ± 0.136 **a**	5.404 ± 0.279	5.306 ± 0.316 **b**
feces	Simpson	D14	0.985 ± 0.003	0.987 ± 0.003 **a**	0.976 ± 0.009b	0.975 ± 0.01 **b**
feces	ACE	D28	1826.3 ± 318.5	2025.9 ± 249	2101.4 ± 309.7	1933.3 ± 250.6
feces	Chao1	D28	1822.1 ± 319	2021 ± 252.3	2107 ± 313.9	1927.8 ± 248.7
feces	Fisher	D28	333.4 ± 67.7	369.9 ± 45	374.2 ± 49	347 ± 51.7
feces	InvSimpson	D28	62 ± 15.2	63.1 ± 8	62.4 ± 21.6	58.8 ± 9.9
feces	Observed	D28	1672 ± 305	1870.9 ± 228	1930.9 ± 310.4	1786.7 ± 246.5
feces	Shannon	D28	5.48 ± 0.275	5.589 ± 0.158	5.551 ± 0.227	5.503 ± 0.198
feces	Simpson	D28	0.983 ± 0.004	0.984 ± 0.002	0.982 ± 0.005	0.983 ± 0.003
cecum	ACE	D28	329.4 ± 136.4	397.5 ± 39.3	387.4 ± 93	440.3 ± 66.1
cecum	Chao1	D28	326.3 ± 143	399.1 ± 38.7	387.1 ± 103.6	457.1 ± 58.9
cecum	Fisher	D28	58.6 ± 19.9 **b**	73.8 ± 13.9	76.3 ± 13.1	83 ± 13.9 **a**
cecum	InvSimpson	D28	17.9 ± 8.1	24.5 ± 14.3	21.5 ± 8.9	29.2 ± 13.9
cecum	Observed	D28	269.9 ± 151.2	323 ± 64.4	315 ± 107.6	378.6 ± 92.7
cecum	Shannon	D28	3.76 ± 0.638	4.106 ± 0.333	4.122 ± 0.29	4.302 ± 0.445
cecum	Simpson	D28	0.929 ± 0.043	0.952 ± 0.015	0.948 ± 0.017	0.959 ± 0.017

Data analyzed from the pig fecal and cecal samples (n=7 per group). For the fecal microbiota, two timepoints are available (14 and 28 d). A, B $P<0.01$; a, b $P<0.05$.

Data concerning beta diversity are presented in figure 9. Bray-Curtis dissimilarities measured in cecal content collected at 28 d revealed no effects of treatments in conditioning across-samples microbial composition (Fig. 9, C). Fecal samples collected at 14 d displayed a significant separation due to treatment*time interaction (P<0.01, Fig.9 A). Furthermore, no effects of treatments were evidenced from PERMANOVA performed on fecal samples collected at 28 d (Fig. 9, B).

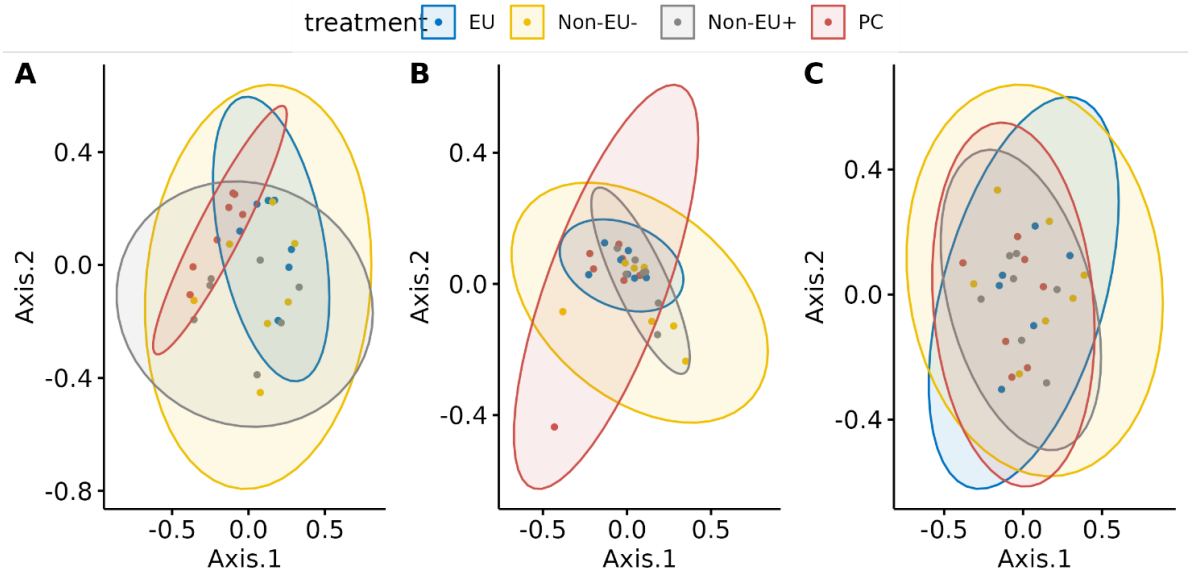

Figure 9. Beta diversity data referred to fecal samples collected at 14 d and 28 d (A, B respectively) and cecal samples collected at 28 d (C).

Differential abundances in cecal content

Differential abundances at genus level for cecal content samples (28 d) are displayed in Figure 10. EU cecal samples showed a higher abundance of *Succinivibrio*, *Prevotellaceae* Ga6A1 and *Campylobacter* than PC ($P<0.05$). Moreover, a reduction in terms of *Fusicatenibacter*, *Erysipelotrichaceae* UCG-003 and *Ruminococcaceae gauvreauii* group was detected in EU vs PC ($P<0.05$). In addition, *Treponema* 2, *Ruminococcaceae* UCG-005, *Roseburia*, *Prevotellaceae* UCG-003 and *Campylobacter* were more abundant in Non-EU- vs PC ($P<0.05$). However, a significative reduction of *Catenibacterium* and *Blautia* was detected in Non-EU- when compared to PC ($P<0.05$). Non-EU^{+} cecal samples highlighted a higher abundance of *Ruminococcaceae* NK4A214, *Prevotella* 7, *Coprococcus* 1 and *Blautia* when compared to PC ($P<0.05$). Finally, a reduction in *Fibrobacter* abundance was detected in Non-EU+ vs PC ($P<0.05$).

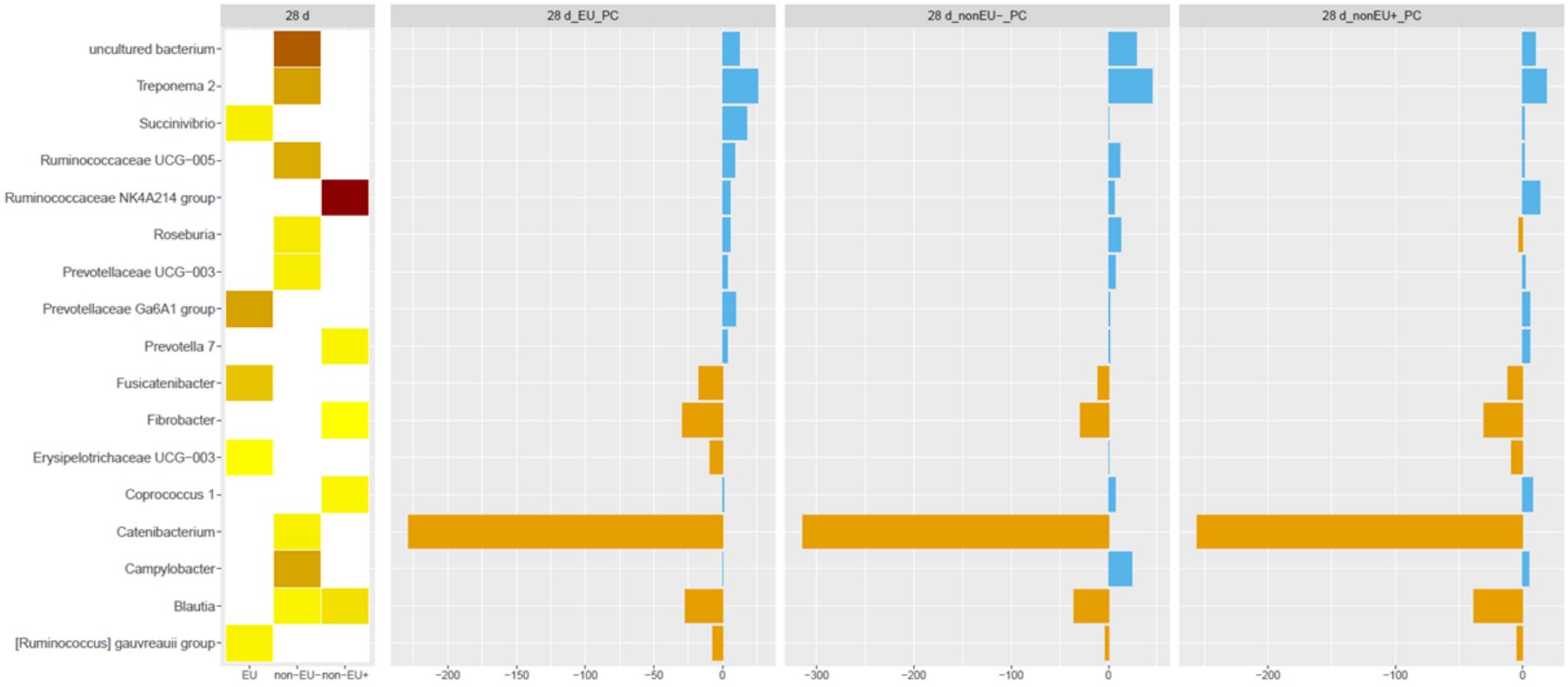

Figure 10. Differential abundances determined in cecal samples collected at 28 d. Significance level was considered between P<0.05 (light yellow) and P<0.01 (dark brown).

Differential abundances in fecal samples

Genus differential abundances of fecal samples collected at 14 and 28 d are presented in figure 11 and figure 12. At 14 d, EU evidenced a higher abundance of *Prevotella* 9, Prevotellaceae (UCG-001 and NK3B31), *Lachnospiraceae* (UCG-001 and NK4A136 group)*, Faecalibaculum* and *Ruminobacter* when compared to PC ($P<0.05$). On the other hand, EU showed a reduction in terms of *Prevotellaceae* (UCG-003, UCG-004), *Clostridium sensu stricto* (6, 13 and 1) and *Sutterella/Parasutterella* in comparison to PC ($P<0.05$). Nevertheless, *Prevotellaceae*. Moreover, a more contained modulation of *Ruminococcaceae* and *Lachnospiraceae* genera was detected in Non-EU^- when compared to PC at 14 d. Furthermore, Non-EU^- highlighted a slightly lower abundance of *Escherichia-Shigella* and a lower presence of *Prevotellaceae* (UCG-001, UCG-003 and NK3B31 group) in comparison to PC ($P<0.05$). Comparing Non-EU^+ to PC at 14 d revealed a similar trend, evidencing a lower abundance of *Prevotellaceae*, *Clostridium sensu stricto* and *Sutterella* genera than PC ($P<0.05$). At 28 d Non-EU- and Non-EU+ showed profuse similarities in terms of abundances of the significantly affected genera, as evidenced by figure 13 and 14.

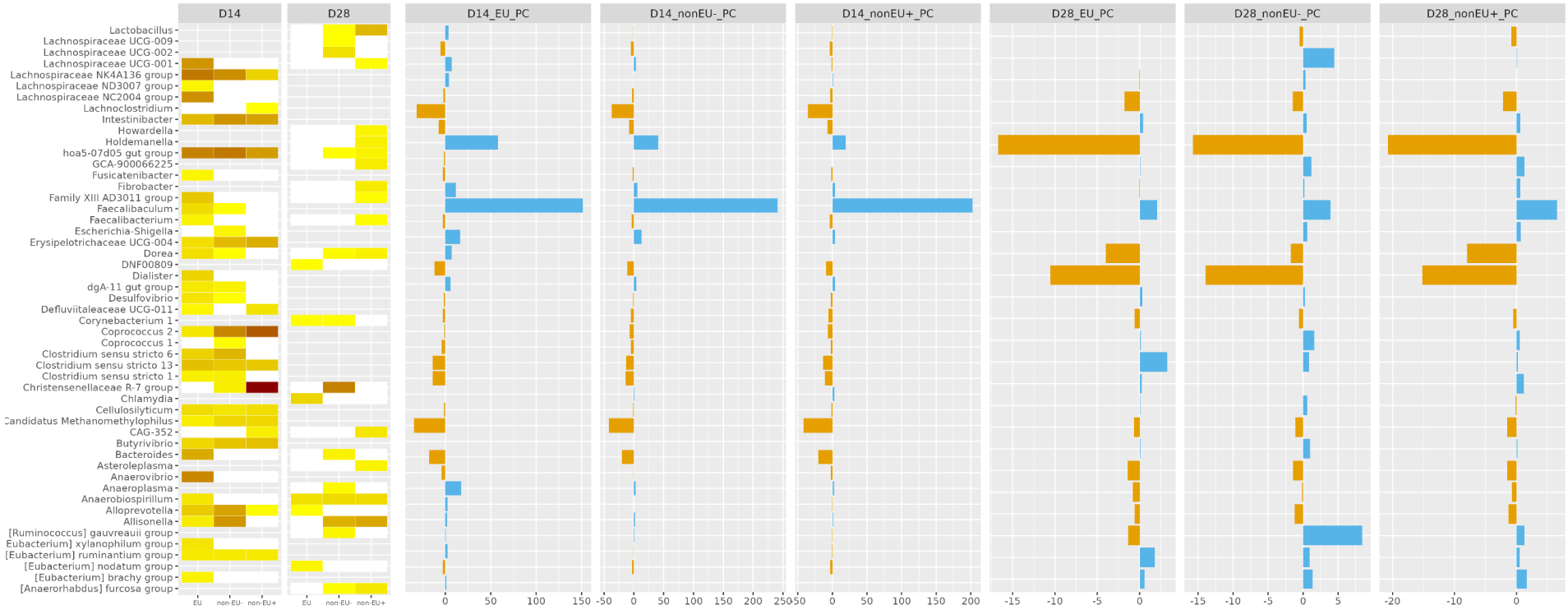

Figure 11. Differential abundances determined in fecal samples collected at 14 and 28 d. Significance level was considered between P<0.05 (light yellow) and P<0.01 (dark brown). Detected genera from A to M (excluded) initials.

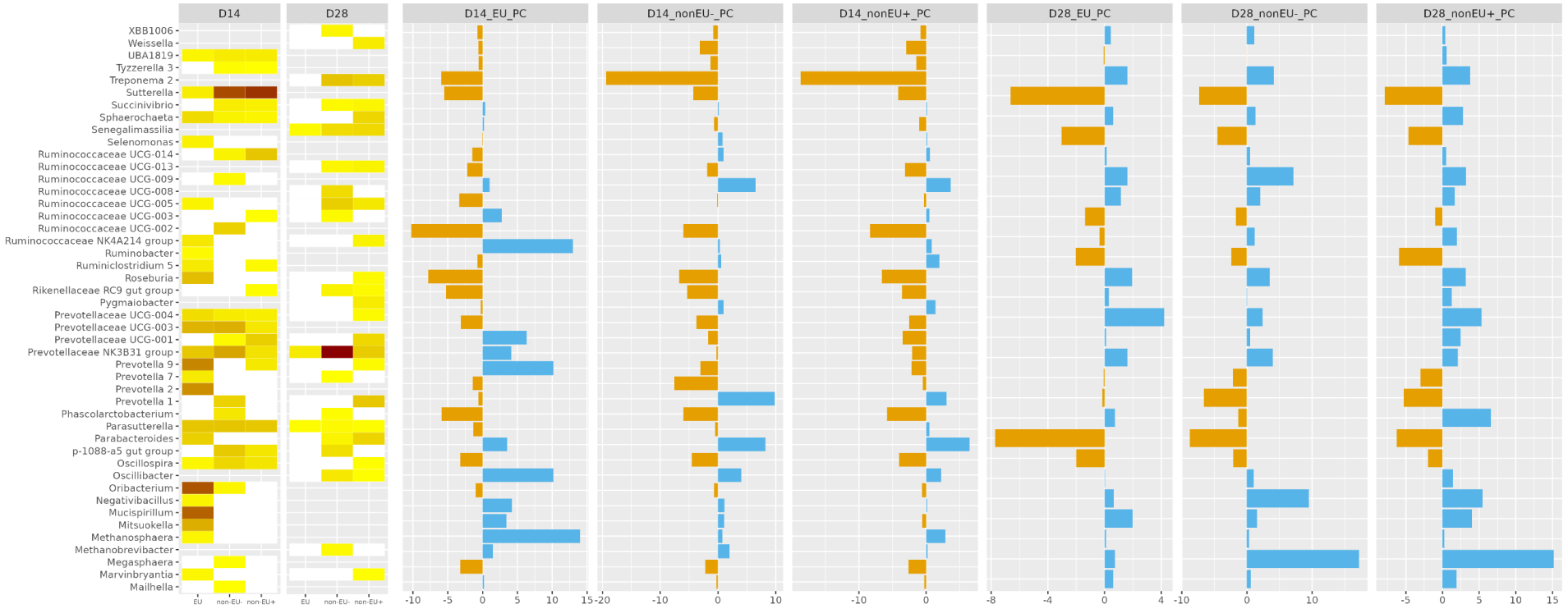

Figure 12. Differential abundances determined in fecal samples collected at 14 and 28 d. Significance level was considered between P<0.05 (light yellow) and P<0.01 (dark brown). Detected genera from M to Z initials

Discussion

The aim of this study was to investigate the possibility to optimize zinc and copper dietary administration through Pot-ZnO and Cu_2O in order to ensure optimal immunity, gut health and microbiota during piglets weaning transition.

In our study, fecal score was monitored daily during the entire trial period. The registered fecal score revealed significant differences between PC and EU only at 4 d on trial. Fecal score was not conditioned by the treatments during the remaining days, and the lack of significative differences among EU, Non-EU^+ and Non-EU^- are in accordance with the available literature. Indeed, Peng et al. (2020) registered a similar diarrhea rate when comparing a PC group of piglets fed 3000 mg/kg of ZnO and two other groups fed 750 mg/kg and 1,500 mg/kg of Pot-ZnO respectively during a trial lasted 14 d. In addition, 200 and 300 mg/kg of Pot-ZnO evidenced similar or better fecal consistency at 7 d and during the late weaning phase in comparison with the administration of 3000 mg/kg of conventional ZnO (Cho et al, 2014).

Morales et al. (2012) reported the capacity of Pot-ZnO in ameliorating performances of weanling piglets in medium-low sanitary conditions when administered at 150 mg/kg, within European limits. Bonetti et al. (2021) highlighted the capacity both to increase or maintain performances of piglets with Non-European concentrations of ZnO administered through different formulations in comparison with pharmacological ZnO dosages. Our results confirmed the possibility to obtain similar performance when comparing PC with EU, Non-EU- and Non-EU+.

Serum pro-inflammatory cytokines represent an important marker of intestinal health and the starting point of innate and adaptive immunity after weaning (Sciascia et al, 2023). Furthermore, pro-inflammatory cytokines could have a role in favouring the intestinal barrier disrupt due to a direct action on junction cells or proteolysis, conditioning the intestinal permeability (Schnoor et al, 2015). IL-6 and IL-1β were previously found to be conditioned by different zinc and copper supplementation levels. Furthermore, zinc deficiency has been linked to an increased IL-6 and IL-1β expression and their signalling pathways (Gammoh et al, 2017). Moreover, IL-6 modulates the transporter Zip14 in the liver, which has been previously linked to hypozincaemia during the acute phase response (Liuzzi et al, 2005). On the other hand, Forouzandeh et al. (2022) underlined that elevated concentrations of Cu have been found to regulate cytokines levels with marked effects on the inflammatory and immune response. Briefly, Long et al. (2017), highlighted the reduction of the relative protein expression of IL-1β in jejunum and IL-6 in ileum samples of piglets fed 500 mg/kg of Pot-ZnO when compared to a positive control group fed 3000 mg/kg of conventional ZnO at 28 d after weaning. As reported by Celi et al. (2019), cytokines analyzed through immuno-assays could represent a valid tool to depict the inflammatory status of the animal. The authors clearly reported that IgA represents an important defence tool for the host to counterattack early infections. Moreover, serum IgA could be considered as an indirect marker of intestinal health. Indeed, serum IgA levels could be related to intestinal mast cell activity and intestinal junctions disrupt during the weaning phase (Levast et al, 2010). In addition, Peng

et al. (2020) studied the effect of the administration of 750 and 1500 mg/kg of Pot-ZnO on serum IgG and IgM levels at 14 d in early weaned piglets. Briefly, the author reported a lack significant differences in serum IgG and IgM when comparing the group fed 750 mg/kg of porous zinc, NC, and PC (fed 3000 mg/kg of conventional ZnO). On the other hand, IgG levels where not different between PC and the group fed 1500 mg/kg of porous zinc oxide but differed when comparing the same group with a negative control. In our case, none of the treatments had effect on circulating pro-inflammatory cytokines level at 14 d. Focusing on immunoglobulins, despite a lack of significance in IgG and IgM levels, the administration of 300 ppm of Pot-ZnO and 140 ppm of Cu_2O showed a higher level of serum IgA when compared to PC. These results underlined the possible role of the tested compounds in modulating the immune response of the animal in challenging situations.

Lipid peroxidation can be exacerbated during the inflammatory processes, and antioxidant defences could limit the cell damage induced by ROS (Szczeklik et al, 2018). Zn is a well-known essential component of many proteins involved in the regulation of the antioxidant status. On the other hand, Cu could act as both antioxidant and prooxidant. Furthermore, changes in Zn and Cu ions homeostasis could favour oxidative stress due to reactive species production enhancement (Osredkar et al, 2011). Moreover, in mice models it has been reported that variation in the Zn/Cu ratio due to trace elements bioavailability could have a role in modulating serum antioxidant status of the animal through SOD and MDA levels changes (Mackenzie et al, 2011). In addition, Liu et al. (2020) revealed that variating the source of Zn has different effects on serum total antioxidant capacity (T-AOC). In particular, the author reported higher T-AOC values in serum samples of piglets fed more bioavailable and organic sources of Zn (100 mg/kg) at 35 d from weaning in comparison with the administration of 100 mg/kg of $ZnSO_4$. Furthermore, pharmacological levels of the tested monovalent copper oxide source (250 mg/kg of Cu) were previously found to not modulate important components of the serum antioxidant status (MDA, SOD and GSH-Px) at 28 d after weaning (Forouzandeh et al, 2022). in our study EU, Non-EU^+ and Non-EU^- showed no differences with the PC group (2500 mg/kg of ZnO) regarding the antioxidant status of the animals 14 d after weaning as depicted by T-AOC and D-ROMs values. DAO and L-lactate have been previously proposed as circulating biomarkers of intestinal permeability in post-weaning piglets (Engelsmann et al, 2023). Moreover, DAO is present in the apical fraction of a developed villus and its increase in the blood stream could be related to the disrupt of the intestinal barrier (Zhao et al, 2014). On the other hand, L-lactate derives from the anaerobe metabolism and could be linked to the develop of specific microbial niches in the gut lumen (Su et al, 2013). The administration of 200 and 500 mg/kg of Pot-ZnO did not decrease serum DAO levels when compared to a pharmacological administration of ZnO (3000 mg/kg) at 28 d after weaning (Peng et al, 2019). On the contrary, Long et al. (2017) showed that 500 mg/kg of Pot-ZnO had a higher DAO level when compared to a PC fed 3000 mg/kg of ZnO. In our study, L-lactate levels were not different among the treatments group. Moreover, Non-EU^- treatment registered a higher DAO level when compared to PC, which is in line with the previous observations. Interestingly, Non-EU^- differed also with Non-EU^+ treatment in DAO concentrations, showing a higher

value at 14 d after weaning. As previously underlined, Zn can suppress Cu ions absorption and efflux through the intestinal barrier, probably due to an enhancement in metallothioneins which favour the sequestration of the trace element in enterocytes (Semrad et al, 1999). Therefore, the limitation of Cu effects supplementation due to high zinc/low copper ratios cannot be excluded.

Zn uptake over the required level could results in a lower efficiency of Zn absorption with a higher excretion in feces (Brugger et al, 2022). As expected, in our study fecal samples collected at 14 d revealed a significant higher concentration of zinc in PC sample when compared to the other groups. This result is in line with other studies concerning the comparison between ZnO pharmacological dosages and lower levels of other ZnO sources. Wang et al. (2018) underlined a reduction in Zn fecal deposition when reducing zinc in the diet from pharmacological levels (3000 mg/kg) to 400 mg/kg and 800 mg/kg. In our study, looking at Cu levels, differences between EU, Non-EU^+ and PC were found at 14 d after weaning, probably due to the absence of Cu supplementation in PC during the first phase. In addition, Non-EU^+ revealed a higher Cu concentration than Non-EU^-. EU showed higher copper values than Non-EU^- at 14 d despite the same Cu supplementation. Feeding high Zn diet could enhance the binding activity of Cu carrier proteins favouring the sequestration of the trace element in the enterocytes. This homeostatic response could explain the lower excretion of Cu in Non-EU^- when compared to both EU and Non-EU^+.

During the second phase of the experiment, lower Zn/Cu ratios were adopted. Adapting Zn and Cu supplementation can be useful to both gain a balanced trace element metabolism and, consequently, a lower output in the environment (Nys et al, 2018). Analyzing cecal content of both trace elements revealed no differences among the experimental groups at 28 d. This result consolidates what Hara et al. (2000) observed: since significant amounts of the trace element are recovered and absorbed in this tract, cecal content can give limited information about trace element metabolism (Hara et al, 2000). On the other hand, zinc concentration in excreta does not differ considering the same supplementation (100 mg/kg) between conventional ZnO and Pot-ZnO (Nielsen et al, 2022). This is expected since the excretion is main drive by the intake, as the trace mineral retention is very low in pigs (Rigolot et al, 2010). Therefore, the results regarding zinc and Cu content in feces at 28 d are in line with literature.

Intestinal alkaline phosphatase (ALP) represents a key indicator to be associate to important functions and changes during the weaning (Lackeyram et al, 2010). ALP is a metalloenzyme in which Zn represent an integral part (Sun et al, 2005). Intestinal ALP may reflect damages of the intestinal barrier as it has been previously linked to tight-junctions modulation and, consequently, gut permeability (Xu et al, 2021). Martin et al. (2013) revealed that jejunal ALP activity increases when administering pharmacological levels of Zn (2500 mg/kg). Alternative forms of zinc were able to increase jejunal ALP activity when compared to sulphates formulation in piglets (Liu et al, 2020). Pot-ZnO showed to modulate intestinal alkaline phosphatase in the distal tract of the intestine when administered at 220 mg/kg in comparison to 110 mg/kg of conventional ZnO (Wang et al, 2018). Furthermore, Cu ions, may

exert an inhibitory effect towards intestinal alkaline phosphatase (Larsen et al, 1996). In our study intestinal ALP levels were not influenced neither by different dietary Zn/Cu ratios administered through Pot-ZnO and Cu_2O nor by pharmacological levels of ZnO. However, the literature is generally not consistent in reporting Zn/Cu ratio effects on intestinal alkaline phosphatase in weanling piglets. Therefore, further evaluations are needed to clarify the possible interaction of Zn/Cu ratios with intestinal ALP.

Secretory IgA are produced in the lamina propria by plasma cells. Briefly, when secretory IgAs are released, they bind antigens inhibiting the proliferation and pathogenic effect of potentially harmful bacteria, contributing markedly to host defence (Wu et al, 2013). Alternative forms of Zn have been linked to enhanced secretory IgA production in avian species and in the jejunal mucosa of weanling piglets (Chang et al, 2023; Shen et al, 2014). In our study, PC showed a higher concentration of IgA in the jejunal mucosa than Non-EU^- but not when compared to EU and Non-EU^+, outlining the similar mucosal IgA concentration between a balanced Zn/Cu administration through potentiated ZnO and monovalent Cu and a pharmacological ZnO dosage. Zn can modulate stimulate gut-associated lymphatic tissue (GALT) immune cells activity in producing local cytokines that can further influence secretory IgA production (Chai et al, 2014). Furthermore, an indirect effect of Cu in conditioning local immunity by acting on pathogens and microbial composition seems more probable (Broom et al, 2021). Therefore, given the observed results limiting Zn excesses may indirectly favor the action of Cu in conditioning gut local immunity.

The interplay between bioavailable trace elements, gut barrier and microbiota could affect microbial niches composition in the gut lumen. Zinc-binding proteins from microorganisms can range from 5% to 6% of the total bacterial proteins produced in the gut lumen (Pajarillo et al, 2021). Therefore, it is reasonable to think that the composition of the microbial core can change due to the bioavailability of trace elements (Gielda et al, 2012). High dietary concentrations of zinc can lead to an imbalanced development of the gut microbiota. In particular, Starke et al. (2014) revealed that feeding weanling piglets 2425 mg/kg of Zn (conventional ZnO) reduced *Enterobacteriaceae*, *Escherichia* and *Lactobacillus spp*. Furthermore, the author underlined both lasting (*Enterobacteriaceae*) and permanent (*Lactobacillus spp.*) effects on microbial composition due to high dietary Zn, which was linked to a reduced lactate concentration in digesta. On the other hand, high dosages of Cu administered thorough $CuSO_4$ were previously linked to gram-negative reduction and volatile fatty acids (VFA) production enhancement in gut digesta (Zhang et al, 2019).

In our study, limited changes were detected at cecal level. Alpha diversity evaluation revealed a reduced Fisher's alpha index in PC group when compared to Non-EU- at 28 d. These variations are in contrast with what Wang et al. (2019) highlighted in terms of diversity and richness when comparing 110 mg/kg and 2400 mg/kg of conventional ZnO with 110 and 220 mg/kg of Pot-ZnO for 15 d after weaning. Briefly, the author did not observed difference among the treatments. Furthermore, Long et al. (2023)

detected more marked changes in ileal digesta when administering 500 mg/kg of Pot-ZnO which revealed a lower Chao1, Observed and Simpson indexes than a PC fed 3000 mg/kg of conventional ZnO. On the other hand, the author underlined a lack of significance in colonic digesta when looking at the difference between PC and 500 mg/kg of Pot-ZnO in alpha diversity indexes. However, in our case fecal samples collected at 14 d highlighted a reduction of Observed, Shannon and Simpson metrics in Non-EU^{-} when compared to EU. Furthermore, Beta diversity highlighted a significant separation in terms microbial composition at 14 d among treatments.

Previously, the administration of Pot-ZnO and Cu_2O was linked to the development of a beneficial microbial population. Supplementing 200 and 500 mg/kg of Pot-ZnO linearly increased *Lactobacillus* and decreased *Escherichia Coli* and *Clostridiaceae* counts in weanling piglets digesta in comparison to 3000 mg/kg of conventional ZnO (Peng et al, 2019). On the other hand, Forouzandeh et al. (2022) reported that 250 mg/kg of Cu administered through Cu_2O reduced *Rikenellaceae* and *Holdemanella*, probably favoring the establishment of a balanced microbiota in weaned piglets. In the present study, *Clostridium sensu stricto* 1, 6 and 13 were more abundant in PC than the other groups at 14 d. *Clostridium sensu stricto* genera have been addressed as potential biomarker of intestinal health (Xu et al, 2021). However, even though most of *Clostridium sensu stricto* bacteria are considered as harmless saprophytes, few of them are recognized as potential pathogens (Li et al, 2023). This result evidenced the capability of Pot-ZnO and Cu_2O to act directly on *Clostridiaceae*.

Sutterella has been previously linked to gastroenteric disfunctions, indicated as IgA-degrading bacteria during ulcerative colitis and negatively correlated to anti-inflammatory cytokines such as IL-13 (Kaakoush, 2020). In the present study, *Sutterella* genera abundance was contained by EU, Non-EU- and Non-EU+ treatments in fecal samples collected at 14 d in comparison to PC. Moreover, Non-EU- displayed a moderate modulation of *Escherichia-Shigella* in comparison to PC, which has been previously linked to gut dysbiosis (Zhao et al, 2022). These results are probably linked to the absence of a direct action of high ZnO dosages towards gram-negative bacteria. Furthermore, it is reasonable to conclude that a more balanced Zn/Cu administration through Pot-ZnO and Cu_2O may be related to better control of potential pathogens.

In addition, balancing Zn/Cu ratios through Pot-ZnO and Cu_2O (EU) showed marked effects on *Prevotella* genera while the pharmacological administration of Zn through conventional ZnO (PC) favoured *Prevotellaceae* genera over the other treatments at 14 d. *Prevotella* and *Prevotellaceae* have been linked to beneficial effects such as endogenous enzymes productions for carbohydrates digestion, better growth performances, diarrhea control and the maturation of the mucosal immunity (Amat et al, 2020). Moreover, the results collected at 28 d can be linked to the microbial stability of the gut environment that can be reached from 10 d after weaning (Chen et al, 2017). Therefore, considering the differential abundances data along with the absence of effects in terms of alpha and beta diversity, it is reasonable to assume that the results were conditioned by more stable enterotypes at the end of the trial.

Conclusions

Balancing Zn/Cu ratios through potentiated zinc oxide and monovalent copper oxide revealed the possibility to modulate different parameters of gut health and microbiota. In particular, intestinal permeability marker and local immunity were conditioned by different Zn/Cu ratios. An excess of dietary zinc in Zn/Cu ratio may be related to limitation in gut health parameters due to enhanced sequestration of Cu. Zn/Cu ratios within European levels of inclusion (EU) could represent an ideal compromise to optimize the effectiveness of both trace elements supplementation during the first two weeks after weaning.

Ethical statement

The experimental design and related procedures were revised and approved by the General Directory of Animal Health and Veterinary Drugs of the Italian Ministry of Health after being visioned by the Animal Welfare Committee of the University of Milan (approval code 790_21/PR).

Consent for publication

Not applicable

Data availability

Upon reasonable request, data are available from the corresponding author: raffaella.rebucci@unimi.it

Competing of interest

Not applicable

Funding

Not applicable

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Author contributions

V.B, A.M: Conceptualization. R.R, L.M, P.C, F.B: Methodology. L.M, F.B: statistical analysis. F.B: bioinformatic processing. L.M, R.R: Investigation. L.M, R.R: Data curation. L.M: Writing-original draft preparation. L.M, R.R, V.B., A.M, Y.M, B.C: writing, reviewing, and editing. V.B, A.M, Y.M: supervision.

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chloride dihydrate, Copper(II) oxide, Copper(II) sulphate pentahydrate, Copper(II) chelate of amino acids hydrate, Copper(II) chelate of protein hydrolysates, Copper(II) chelate of glycine hydrate (solid) and Copper(II) chelate of glycine hydrate (liquid) as feed additives for all animal species and amending Regulations (EC) No 1334 / 2003, (EC) No 479 / 2006 and (EU) No 349 / 2010 and Implementing Regulations (EU) No 269 / 2012, (EU) No 1230 / 2014 and (EU) 2016/ 2261 (europa.eu)

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CHAPTER 8: Effects of *Weizmannia faecalis* DSM 32016 on Growth Performance, Gut Microbiota, and Health Parameters in Holstein Friesian Calves

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Brief introduction to the study: Antimicrobial resistance minimization is still a fixed aim worldwide. Nevertheless, antibiotic administration to solve neonatal diarrhea is still widely used in pre-weaning calves. Probiotics have been used for many years to prevent diarrheal disorders and enhanced gut health by avoiding antibiotic prescription. However, incessant research on effective strategies is still needed. *Weizmannia faecalis*, previously known as *Bacillus coagulans*, could represent an ideal tool to enhance gut health of pre-weaning calves thanks to specific functional characteristics. This study gave the opportunity to investigate weaning transition challenges in another species than swine while consolidating the knowledge of gut microbiota dynamic changes during weaning transition. The present results were also presented during an international congress at 75th European federation of Animal Science (EAAP) meeting helded in Florence (Italy, 1st-5th September 2024). The final output of this part of the project consisted in one manuscript which has been submitted in the Italian Journal of Animal Science and, to date, is under revision.

Abstract

Weizmannia-based probiotics (formerly allocated to *Bacillus*) are used to improve performance and intestinal health of monogastric animals worldwide. However, their impact on calves is not well investigated. Therefore, this study objective was to evaluate the effects of *Weizmannia faecalis* DSM 32016 (WF-32016, formerly *Bacillus coagulans*) on growth- and health-relevant parameters in female Italian Holstein Friesian calves. Twenty calves (seven-day-old) were divided into two groups fed a basal diet: a control group (CTR) lacking additional supplementation, and a treatment group supplemented with 10^9 CFU/kg feed of the probiotic strain WF-32016 (PRO). Colostrum samples, collected 6 h and 12 h post-birth, showed IgG levels above 50 g/l. At end of trial (day 56), BW and fecal scores of probiotic-fed calves were significantly improved compared to control animals ($P<0.05$ and $P<0.01$, respectively). Blood analysis indicated significantly higher IgG levels ($P<0.01$) and antioxidant capacity ($P<0.01$) in probiotic-fed calves at day 56. 16S rRNA gene analysis of fecal microbiota demonstrated significant differences in beta-diversity between groups ($P<0.05$). Furthermore, relative abundances of *Lactobacillaceae, Streptococcaceae* and *Lachnospiraceae,* as well as *Ligilactobacillus*, *Limosilactobacillus*, *Bifidobacterium*, *Alloprevotella*, *Blautia* and *Prevotella 9* shifted significantly throughout the trial period ($P<0.05$). Functional predictions of the microbiota revealed a significant increase in the relative abundance of the pentose phosphate pathway (related to glycolysis and biosynthesis) in samples of probiotic-fed calves which was not observed in samples of control animals ($P<0.05$). In conclusion, dietary supplementation of WF-32016 in female calves improved growth performance, diarrhea, immunity, and antioxidant status, and positively modulated the fecal microbiota.

Keywords: Nutrition, Probiotic**,** dairy, Feed additive, gut health

Introduction

Health and future production of dairy herds depend on calf raising practices, therefore, an integrated approach to calf management is crucial in dairy farms. In order to develop passive immunity, it is essential that calves get a sufficient and timely amount of high-quality colostrum (Hammon et al. 2020). In addition, calves exhibit heightened vulnerability to opportunistic colonization by potentially pathogenic bacteria often in response to environmental or managerial stress conditions (Hulbert and Moisá, 2016). Diarrhea occurring within the initial three weeks of life, followed by respiratory diseases, represents the predominant health challenges encountered by calves (Malmuthuge et al. 2015). These diseases are widely recognized as responsible of high morbidity and mortality in pre-weaning calves, leading to significant economic losses both direct, linked to therapies, and indirect, caused by the lack of future productivity (Dubrovsky et al. 2020; Meganck et al. 2015; Timmerman et al. 2005). Historically, antibiotics were extensively prescribed, both to treat common diseases and to enhance productivity and animal growth. Prolonged utilization of these substances has contributed to the emergence of drug-resistant microorganisms, causing a risk to consumer and animal health, and leading to an increasing urge to limit the abuse of antimicrobials (Thames et al. 2012). European legislation on antimicrobial use in farm animals, responding to concerns over antimicrobial resistance (AMR), has been rigorous. A key move was the ban on antibiotic-based growth promoters starting from 2006, aimed at curbing overuse and preventing the emergence of drug-resistant microorganisms. Ongoing measures emphasize responsible veterinary practices, prescription-only access to antimicrobials, and robust monitoring. Therefore, effective measures oriented to a balance between protecting animal health, ensuring food safety, and addressing the global challenge of antimicrobial resistance in the interest of public health are strictly needed (Ferri et al. 2017). Since the administration of antimicrobial is now limited, alternative solutions to assure calves health should be taken into consideration.

The gastrointestinal tract of calves undergoes substantial microbial colonization immediately after birth and then is shaped by environmental and managerial factors. The initial colonization of the gut microbiota in calves is influenced by numerous factors, including colostrum, diet, and environmental exposure (Liu et al. 2019). The intestinal microbiota of calves undergoes significant changes from birth to weaning, with the establishment of a stable community being pivotal for long-terms health and productivity of the animals (Klein-Jöbstl et al. 2019). The dynamic successions of calf gut microbiota and the interactions among specific bacteria have been found to influence calf diarrhea, emphasizing the role of microbiota in calf health (Chen et al. 2022). Understanding the dynamics of gut microbiota establishment of dairy calves during the weaning transition is crucial for improving early-life gut health and developing microbial modulation strategies to enhance calf health (Malmuthuge and Guan, 2017). Feed additives may represent a useful tool to support gut microbiota development to gain a more resilient microbial community and intestinal health (Dill-McFarland et al. 2019).

Different feed additives have been widely considered and commercially explored in livestock production. Probiotics are defined as live microorganisms that, once administered in adequate concentrations, confer a health benefit on the host (Hill et al. 2014). In animal nutrition, probiotics are used as feed supplements to promote beneficial effects for the gut health of the host animal (Gaggìa et al. 2010). In particular, the use of probiotics in calf nutrition has shown promising results in establishing a balanced microbiota in veal calves (Timmerman et al. 2005). Additionally, the supplementation of probiotics during the milk-feeding period has been found to improve the growth performance of Holstein calves (Wang et al. 2023). Probiotics have also been reported to have useful effects, including improved body weight gain, feed conversion, and fecal consistency in newborn calves and piglets (Alawneh et al. 2020; Cangiano et al. 2020). Moreover, supplementing probiotics to young calves has been suggested as an alternative therapeutic option for preventing enteric diseases and reducing the administration of antimicrobials to neonatal calves (Liang et al. 2020). Overall, the use of probiotics in calf nutrition has shown potential benefits in improving growth performance, nutrient digestibility, and overall health (Markowiak and Ślizewska, 2018). *Weizmannia coagulans* and *Weizmannia faecalis* (both formerly *Bacillus coagulans*), are closely related and probiotic bacteria with distinct characteristics that contribute to its efficacy in promoting gut health. In contrast to typical *Bacillus*-based probiotics, *Weizmannia* produces considerable amounts of lactic acid (via homofermentative lactic acid fermentation). The advantages of intestinal lactic acid production are highly divers and include the stimulation of intestinal butyrate production, broad modulation of the immune system, and reinforcement of the intestinal barrier (Duncan, Louis and Flint 2004; Bourriaud et al. 2005; Garrote, Abraham and Rumbo 2015; Ratter et al. 2018). Furthermore, members of *Weizmannia* are gram-positive and form endospores (Hammer, 1915; De Clerck et al. 2004) that bacteria. withstand harsh environmental conditions, such high temperatures, pressure, or gastric acidity. Once the *Weizmannia* spores germinate in the nutrient-rich intestine (Kapse et al. 2019) the bacteria start producing lactic acid and using divers other mechanisms of action, such as direct and indirect pathogen inhibition or digestive enzyme production, to support the gut microbiota, digestion, and overall intestinal health (Halder et al. 2024; Raheem et al. 2021;Zhou et al. 2020). *Weizmannia* is, therefore, used in human medicine and nutrition to supports intestinal health, immune function, and overall well-being in humans (Dolin, 2009; Zhou et al., 2020; Cao et. al., 2020). In this regard, gastrointestinal disorders, including diarrhea, abdominal pain, digestive problems, and overgrowth of harmful bacteria can be reduced due to *W. coagulans* supplementation (Dolin, 2009; Zhou et al., 2020). By acting as an anti-diarrheal agent that supports gastrointestinal integrity and prevents weight loss, *W. coagulans* is also suitable for treating antibiotic-induced gut dysbiosis (Gupta and Maity, 2021; Cao et. al., 2020).

In animal nutrition and production, the effect of *Weizmannia* has been evaluated in poultry and weaning piglets (Parhiszkar et al., 2022, Elleithy et al., 2023, EFSA Panel, 2020). Besides an improved production performance, feed efficiency and intestinal health parameters were positively affected by using *Weizmannia*-based probiotics (Parhiszkar et al., 2022, Elleithy et al., 2023, EFSA Panel, 2020;

Wu et al. 2018; Wang and Gu 2010). However, the impact of *Weizmannia* in calves is not well investigated (Ripamonti et al. 2009). Therefore, further investigations regarding the effect of probiotic *Weizmannia* in calves are needed.

In this study, *W. faecalis* DSM 32016 (WF-32016), attested for safety and efficacy in piglets and poultry, was supplemented in Italian Holstein Friesian female calves. The aim was to evaluate the effects of the probiotic on growth performance, blood parameters, and fecal microbiota composition, with a particular focus on the challenges associated with weaning.

Materials and Methods

Experimental design and animal housing

The trial was performed at Azienda Agricola "Ceradello" (Crotta d'Adda, Cremona, Italy) and based on 20 female Italian Holstein Frisian dairy calves. Calves were monitored by the farm veterinarian from birth on till trial start to ensure that only healthy animals, which did not exhibit any diarrhea symptoms or other signs of disease within their first week of life, were included. No antibiotics were used for prophylaxis or metaphylaxis. Animals showing clinical signs of disease other than diarrhea, within the first month of life were excluded from trial (Turini et al. 2022). Consequently, 20 healthy animals were randomly divided in two feeding groups: control (CTR) and probiotic treatment group (PRO). The trial lasted for 56 days, starting when the calves were 7 days old and concluding when they were 63 days old. Calves were housed in individual units (2.2 x 1.5 m) with straw bedding that was routinely renewed. Units were organized to let calves interact with each other for the entire duration of the trial, in accordance with European Legislation (2008/119/CE). For experimental purposes, calves have been housed individually until 9 weeks of life in compliance with D.Lgs 126/201 which provides that calves older than 8 weeks could not be housed in individual pens, unless under exceptional circumstances.

Animals diet

Calves of both groups, CTR and PRO, received diet based on milk replacer (CMR) and starter feed that were analyzed for their nutrient and chemical composition (Tabel 1 and Table 2). Except of the probiotic supplementation, CTR-diet and PRO-diet were identical. The probiotic inclusion of WF-23016 (Product name: TechnoSpore®; Biochem Zusatzstoffe Handels- und Produktionsgesellschaft mbH, Lohne, Germany) was 10^9 CFU/kg of CMR and starter feed, corresponding to 400 mg of additive per kg feed Two colostrum samples were collected from each dam after delivery to assess the level of immunoglobulins G (IgG). Colostrum was collected in sterile 15 ml-tubes at 6 h and 12 h after birth. Quantification of IgG was performed using a commercial ELISA kit (Astori Tecnica) following manufacturer's instructions within 1.5 h after colostrum collection. Colostrum IgG content confirmed that 96% of colostrum samples were characterized by IgG levels above 50 g/L, indicating a high quality of colostrum (Gulliksen et al. 2008). Within the first 12 h after birth calves were fed 4 L dam's colostrum divided into two equal meals. In particular, the first colostrum was fed between 30 min and 2 h after birth, whereas the second feeding was provided during the following 8 hours (Turini et al. 2020). This process was repeated the second day after birth, using frozen aliquots of dam's colostrum. Frozen colostrum was thawed at 39 °C to avoid overheating and immunoglobulins denaturation (Sandra Godden, 2008). Complete colostrum intake was monitored by an expert farm operator. Starting at day 3 of life, calves were fed two times a day (7:00 AMand 3:30PM) with 2 L transition milk (4 L in total). At day 7 of life (trial start), calves were transitioned to a concentrated milk replacer (CMR), provided at a temperature of 39°C. Lactation curve required a constant concentration of 130 g/l of CMR. The administration of CMR started at 4 L/day (trial week 1 – 2; day 7 - 21 of life)) and gradually increase to 8 L/day (trail week 3 – 6; day 22 – 48 of life)). Beginning at trial week 7 (day 49 of life), CMR was reduced to 6 L/day till end of trial (trial day 56; 63 day of life). CMR was administered using slow-suction drip buckets. CMR of CTR- and PRO-diet were prepared routinely using different blenders to avoid cross contamination. Calves had free access to water and pellet starter feed was introduced on the second day of life, with leftovers quantified and documented daily. . Nutritional values of the diets have been calculated in accordance with the nutrient requirements recommended by NRC (2021) for dairy calves. Compositional analysis of starter feed and CMR were performed at the Laboratories of the Department of Veterinary and Animal Sciences (University of Milan – Via dell'Università 6, Lodi). Analyses were performed as follows: Dry Matter (DM) was determined by drying milk powder at 65°C for 24 h (AOAC method 930.15); Crude Protein (CP) was measured using the Kjeldahl method (AOAC method 2001.11); Ether Extract (EE) was assessed by ether extraction with the Soxtec system (AOAC method 2003.05); Crude Fibre (CF) was analyzed using AOAC method 978.10; and ash content was obtained by combusting samples in a muffle furnace at 550 °C (AOAC method 942.05). CF analysis was limited to the starter feed, as the fibre content in CMR was below the detection limits (0.30-0.50%; Fahey et al. 2019). Analyses was based on five samples of CMR and five samples of starter feed (Table 1 and Table 2).

Table 1. Nutritional and chemical analyses of CMR.[1]

Parameters[2] (% as fed)	Declared composition[3]	Chemical analyses
DM, %	-	96.50 ± 1.70
CP, %	22.00	22.50 ± 2.10
EE, %	20.00	18.00 ± 1.87
CF, %	0.20	-
Ash, %	6.90	7.7 ± 1.05

[1] Data are presented as mean ± standard deviation (n=5). CMR composition consisted in 50% skimmed milk powder, mixture of vegetable oils, products and by-products of cereal grains, organic acids, vitamins, minerals, DL-methionine and L-lysine (declared by the producer, Zoogamma S.p.A., Strada Borgosatollo 5/A, 25016 Ghedi, Italia).

[2] DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fibre.

[3] Additives per kg : Vitamin A 25000 IU; Vitamin D3 3000 IU; Vitamin E 80 mg; iodium: 1 mg; manganese: 35 mg; zinc: 120 mg Se: 0.3 mg; Cu 10 mg/kg; Fe 100 mg/kg.

Table 2. Nutritional and chemical analysis of starter feed[1]

Parameters[2] (% as fed)	Declared composition[3]	Chemical analyses
DM, %	-	89.00 ± 1.20
CP, %	17.00	16.74 ± 1.75
EE, %	4.00	4.00 ± 0.90
CF, %	10.64	9.81 ± 1.37
Ash, %	8.00	7.57 ± 1.49

[1]Data are presented as mean ± standard deviation (n=5). Starter feed composition consisted in wheat bran, maize, wheat mince, sunflower seed extraction flour, alfalfa flour, wheat distillates, calcium carbonate, rice husk, beet molasses, soybean flour (48% CP), dried and ground carob beans (declared by producer, Calf Feed, Ferraroni Mangimi, Via Casalmaggiore 18, 26040 Bonemerse, Italia)

[2]DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fibre.

[3]Additives per kg: Vitamin A 10710 IU; Vitamin D3 3000 IU; Vitamin E 24 mg; Vitamin B12 0.036 mg; Beta-carotene 1.20 mg; Cu 17.50 mg; Fe 31.50 mg; I 1.40 mg; Mn 84 mg; Se 0.35 mg; Zn 105 mg/kg.

Growth performance, morphometric measurement, and fecal score

Individual body weight (BW) was recorded at day 7, 35 and 63 days of life (trial day 0, 28, and 56) by using a 250 x 70 cm electronic weighting balance (CIMA control cattle, CIMA, Correggio, Reggio Emilia, Italy). Average daily gain (ADG) was calculated for three phases, day 0-28, day 28-56, and day 0-56. Additionally, biometric parameters were measured using a calibrated meter ad included body length, heart girth, withers height and hip width (Hoffman, 1997). CMR and starter feed consumption were recorded daily to assess the average dry matter intake (DMI). Feed conversion ratio (FCR) was calculated on the total amount of ingested dry matter. Health conditions of calves were routinely checked. Fecal score evaluation was conducted through a 4-points consistency scoring scale: 0 = firm; 1 = soft; 2 = runny; 3 = watery as reported by Renaud et al. (2020).

Whole blood analysis, plasma metabolic biomarkers, IgG, and serum antioxidant capacity

At trial day 0, 28, and 56, blood was sampled from jugular vein using 10 ml tubes armed with a 18G needle (VACUETTE®, Greiner Bio-One GmbH). Blood samples were taken in the mornings between 8:00 AM and 12:00 PM. Serum and plasma aliquots were obtained through centrifugation at 3000 rpm for 15 min at 4 °C and stored at −20 °C until analysis. Non-coagulated blood was Analyzedanalyzed for red blood cell (RBC), haemoglobin (HB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), and platelet count (PLT) by using an automated veterinary haematology analyzer (Synchron clinical CX 5 Delta, Beckmann Coulter Italia, Milan, Italy).. Furthermore, glucose, non-esterified fatty acids (NEFA), and beta-hydroxybutyrate (BHB) where determined in plasma aliquots and differential leukocyte count was performed microscopically on Giemsa-stained blood film applying the cross-sectional method (Mohri et al. 2007). Plasma IgG and serum total antioxidant capacity (T-AOC) were determined by using colorimetric assays and ELISA kits (Società Italiana Chimici s.r.l).

Fecal samples evaluation and sequence library preparation

Fecal samples were collected directly from the rectal ampulla from all 20 calves at trail day 0, 28 and 56 d and stored at −80 °C until DNA extraction. DNA extraction was according to indications depicted by Cremonesi et al. (2022), using the QIAmp Fecal Pro kit (Qiagen, Hilden, Germany), following to the manufacturer's protocol. Extracted DNA was assessed for quality and quantity using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and stored at −20 °C until further use. Bacterial DNA was amplified using primers targeting the V3-V4 hypervariable regions of the 16S rRNA gene (Caporaso et al. 2011). For PCR amplification, a 12.5 µL of KAPA HIFI Master Mix 2× (Kapa 344 Biosystems, Inc., MA, USA) and 0.2 µL of each primer (100 µm) were added to 2 µL of DNA (5 ng/µL). The PCR was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific). Samples were denatured at 95 °C for 3 min, followed by 25 cycles with a denaturing step at 98 ◦C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, 351 USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2×250-base paired-end reads.

16S rRNA gene sequence analysis

The reads obtained by 16S sequencing were analyzed using QIIME (v. 1.9.0). Clustering filtered reads into phylotypes at 97% identity level and discarding singletons as possible chimeras. Taxonomic assignment was performed via the RDP (Caporaso et al. 2010; Wang et al. 2007) classifier against the SILVA database (release 138, Pruesse et al. 2007). Bacterial diversity analysis was computed through QIIME.: Alpha-diversity was obtained using different metrics (Shannon's diversity, Chao1 diversity index, Observed species) and Faith's phylogenetic diversity index (PD whole tree) wheras beta-diversity was evaluated through the unweighted and weighted UniFrac distances. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was utilized to map 16S rRNA gene amplicon sequences to known reference genomes, allowing for the prediction of gene taxa abundances in host-associated microbial communities (Langille et al., 2013; Douglas et al., 2020). This bioinformatics analysis was performed using the PICRUSt2 software and the MetaCyc ontology annotation (Douglas et al. 2020) and enabled the prediction of the functional properties of the fecal microbial communities in calves.

Statistical analysis

Growth performances, morphological measurements, plasma glucose, NEFA, BHB, IgG and serum TAC were evaluated through a MIXED procedure of SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA) accounting for the effect of treatment, time, and their interaction. Post-hoc evaluation for multiple comparisons was performed through a Tukey test. Data are presented as mean ± standard error mean (SEM). Data collected at the first time point were used as covariate.

As per the microbiota pipeline, a Mann–Whitney U-test was applied to assess alpha-diversity statistical differences between CTR and PRO, while the Permanova test (adonis function) from the package "vegan" (v 2.6-6.1) was used to analyze beta-diversity differences (Oksanen et al. 2024). Taxonomic statistical analyses were performed in R (v. 4.3.3, The R Project): the Wilcox test was implemented for all comparisons between PRO and CTR groups, while differences between groups across time-points were assessed via Kruskall-Wallis test and followed by Dunn's- test with the Bonferroni correction. Spearman ρ correlation analysis was performed to evaluate the relationship between taxonomic variations and the metabolic pathways. The predicted pathway analysis was carried out using MaAslin2 multivariate analysis via the "ggpicrust2" R package. P values ≤ 0.05 were considered as statistically significant.

Results

Effect of WF-32016 on growth performance and feed conversion

The administration of WF-32016 significantly affected the growth performance of calves (Table 3). In particular, the final BW (at trial day 56) was significantly higher in the PROgroup in comparison to the CTR group when considering the interaction among groups ($P<0.01$) and time (PRO: 75.55 ± 4.55 kg; CTR: 67.90 ± 6.09 kg; $P<0.05$). Furthermore, the probiotic treatment significantly enhanced ADG($P<0.01$), although there was no significant interaction with time ($p=0.86$) . Average daily milk intake of the PRO group showed a tendence of increase towards significance compared to the CRT group ($p=0.053$). FCR calculations indicated a significantly improved feed conversion in the PRO group compared to CRT ($P<0.05$)

Table 3. Growth performance, feed intake, and feed conversion of calves in control (CTR) and probiotic-treated (PRO) group.[1]

	Group			*p*-value		
Parameter	CTR	PRO	SEM	Time	Group	Time*Group
BW (kg)						
0 d	41.11	41.30	1.28	<.0001	<0.01	0.021
28 d	49.75	52.85				
56 d	67.90[b]	75.55[a]				
ADG (kg/d)						
0-28 d	0.308	0.412	0.051	<.0001	<0.01	0.86
28-56 d	0.648	0.811				
0-56 d	0.478	0.612				
DMI (kg/d)						
0-28 d	1.105	1.094	0.088	<.0001	0.240	0.53
28-56 d	1.724	1.914				
0-56 d	1.415	1.504				
FCR						
0-28 d	4.23	3.06	0.33	0.03	0.04	0.55
28-56 d	2.93	2.53				
0-56 d	3.02	2.51				

[1] Values are the arithmetic means at trail day 0, 20, and 56 or phase-wise. SEM, standard error of mean. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake on a dry matter basis; DMI, dry matter intake; FCR, feed conversion ratio. Different letters indicate significant differences between groups (P<0.05).

Effect of WF-32016 on biometric parameters and fecal scores

Body length, heart girth, withers height, and hip width were not significantly different between groups (Table 4). However, a tendence of increase towards significance was observed in the PRO group in comparison to CRT when considering the group factor for wither height parameter (P=0.06).

Table 4. Biometric parameters of calves in control (CTR) and probiotic-treated (PRO) group[1]

	Group			*p*-value		
Parameter	CTR	PRO	SEM	Time	Group	Time*Group
(cm)						
Body length						
0 d	65.50	67.70	1.16	<.0001	0.16	0.79
28 d	72.20	73.60				
56 d	79.90	80.50				
Heart girth						
0 d	78.90	78.70	0.98	<.0001	0.24	0.48
28 d	86.10	87.00				
56 d	97.80	100.00				
Withers						
height						
0 d	77.20	77.50	0.93	<.0001	0.06	0.44
28 d	82.10	83.60				
56 d	87.20	89.90				
Hip width						
0 d	18.10	17.80	0.38	<.0001	0.35	0.13
28 d	19.60	19.60				
56 d	20.90	22.10				

[1] Values are the arithmetic means at trail day 0, 28, and 56. SEM, standard error of mean.

Fecal scoring throughout the first two trial weeks (day 7 – 21 of life) indicated a significant effect due to probiotic treatment (Figure 1A; P<0.01). However, daily time points of measurements were not significantly different between groups (Figure 1B). At trial day 14, animals of CRT group reached a similar fecal score like observed in the PRO group.

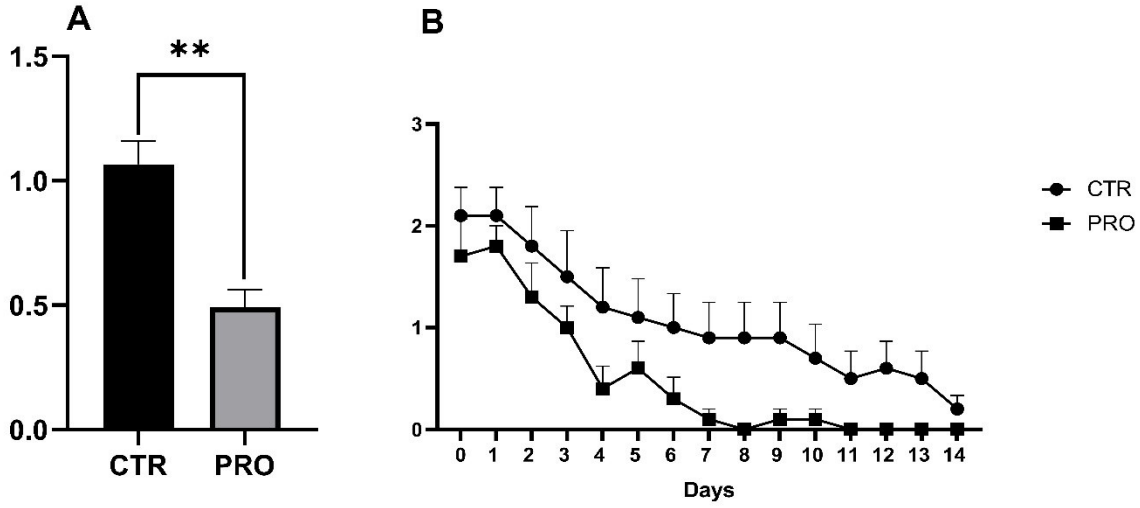

Figure 1. Fecal scores of calves in control (CTR) and probiotic-treated (PRO) group during the first two weeks of trial. Data are presented as the average across all data points per group (A) and time-resolved measurements (B)**.** B, values represent arithmetic means (n = 10), with error bars indicating the standard error of the mean (**P<0.01).

Effect of WF-32016 on whole blood parameters

No significative differences between groups were found regarding red blood cells (RBC, Figure 2 A), haemoglobin (HB, Figure 2 B), haematocrit (HCT, Figure 2 C), mean corpuscular volume (MCV, Figure 2 D), mean corpuscular haemoglobin (MCH, Figure 2 E), mean corpuscular haemoglobin concentration (MCHC, Figure 2 F), red cell distribution width (RDW, Figure 2 G), and platelet count (PLT, Figure 2 H). Furthermore, white blood cell (WBC) count (Figure 3 A) and differentiated leukocytes (Figure 3 B-H) evaluation revealed no differences among groups.

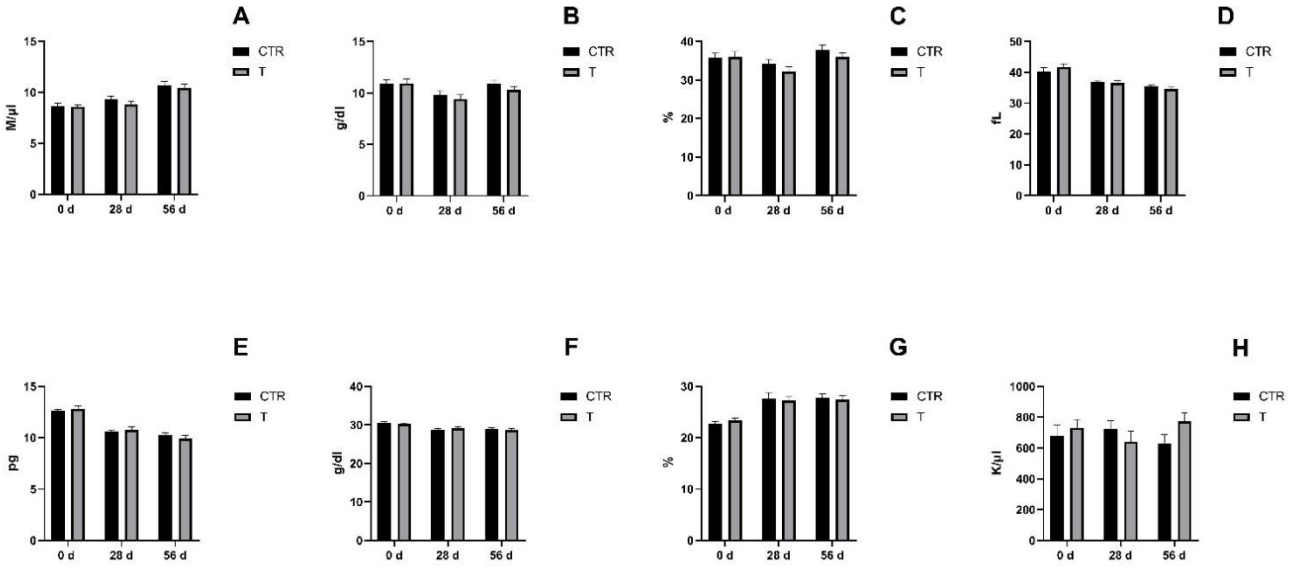

Figure 2. Red blood cell (RBC, A), haemoglobin (HB, B), haematocrit (HCT, C), mean corpuscular volume (MCV, D), mean corpuscular haemoglobin (MCH, E), mean corpuscular haemoglobin concentration (MCHC, F), red cell distribution width (RDW, G), and platelet count (PLT, H) analysis of calves in control (CTR) and probiotic-treated (PRO) group. Values are the arithmetic means (n=10) at trail day 0, 28, and 56. Error bars indicate the standard error of mean (SEM).

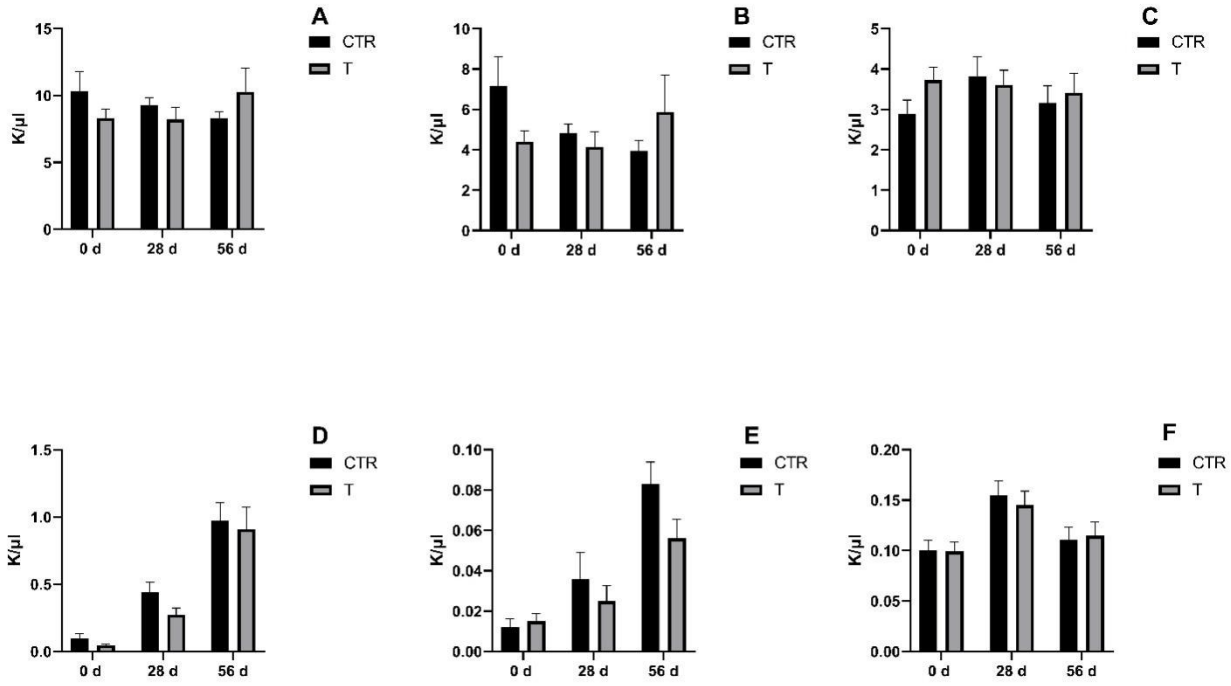

Figure 3. White blood cell (WBC, A) count, neutrophils (B), lymphocytes (C), monocytes (D), eosinophils (E), basophils (F) analysis of calves in control (CTR) and probiotic-treated (PRO) group. Values are the arithmetic means (n=10) at trail day 0, 28, and 56. Error bars indicate the standard error of mean (SEM).

Effect of WF-32016 on plasma metabolic parameters, IgG, and serum antioxidant capacity

Plasma glucose, NEFA, and BHB concentrations were not significantly affect by the probiotic treatment (Figure 4 A-C). In contrast, plasma immunoglobulins G level, at trial day 56 d, was significantly enhanced in animals of the PRO group in comparison to CTR group animals (18.98 ± 2.99 mg/ml vs 15.22 ± 2.40 mg/ml P<0.01; Figure 4 C). Furthermore, serum antioxidant capacity gradually increased in in PRO animals over time until reaching a significant difference, at day 56, when comparing to CTR animals (581.20 ± 52.36 mmol HClO/ml vs 390 ± 59.94 HClO/ml; P<0.01, Figure 5).

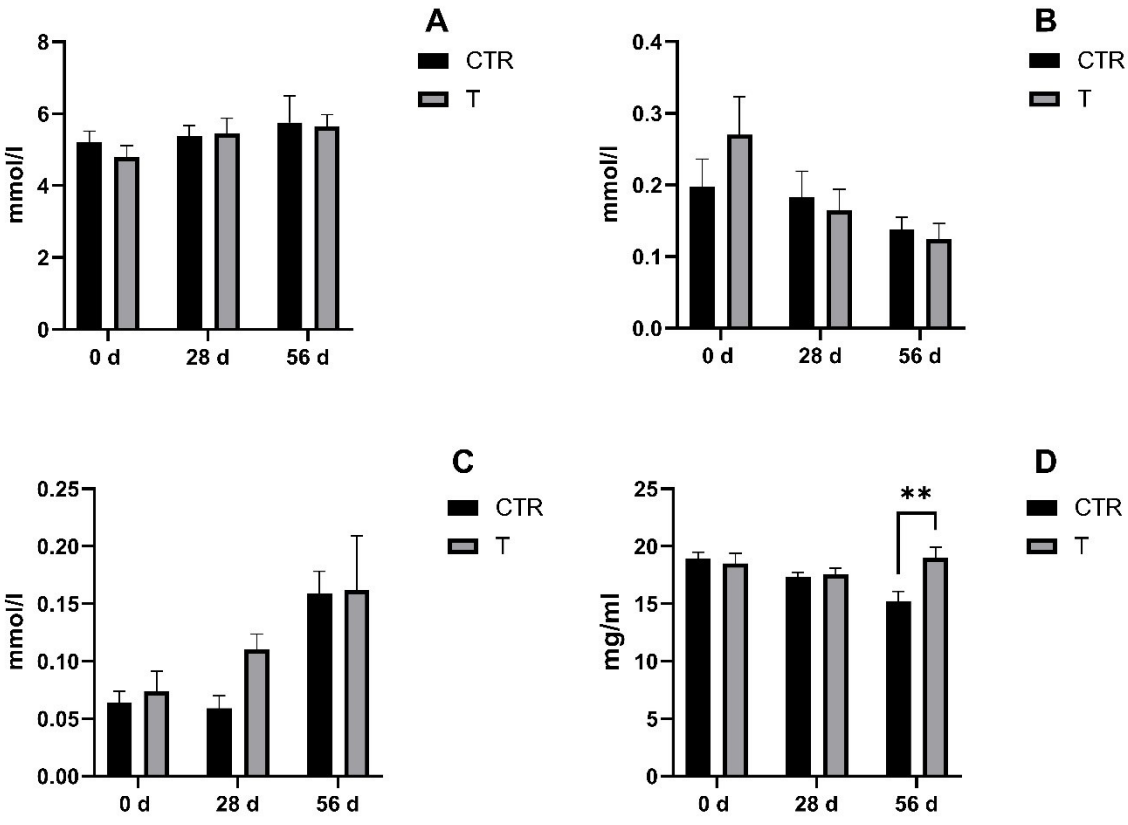

Figure 4. Figure 4. Plasma Glucose (A), non-esterified fatty acids (NEFA, B), beta-hydroxybutyrate (BHB, C), and immunoglobulin G (IgG, D) analysis of calves in control (CTR) and probiotic-treated (PRO) group. Values are the arithmetic means (n=10) at trail day 0, 28, and 56. Error bars indicate the standard error of mean (SEM). Asterisks indicate significant differences between groups (*, P ≤0.05; **, P<0.01).

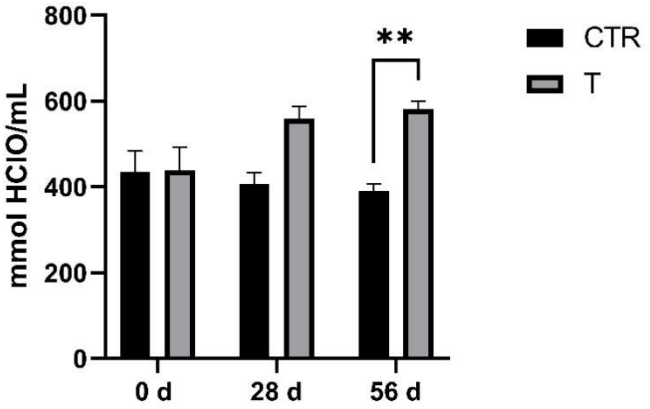

Figure 5. Total antioxidant capacity (T-AOC) analysis of blood serum of calves in control (CTR) and probiotic-treated (PRO) group. Values are the arithmetic means (n=10) at trail day 0, 28, and 56. Error bars indicate the standard error of mean (SEM). Asterisks indicate significant differences between groups (**, $P<0.01$).

Effect of WF-32016 on bacterial diversity and microbiota composition in fecal samples

A16S rRNA gene analysis indicated that alpha-diversity increased in both groups over time with significant differences between trial day 0 and day 56 (Shannon index, Observed species, and PD whole tree metrics: p=0.015; Figure. 6 A). However, no significant differences regarding alpha-diversity were detected between CTR and PRO group.). Similar results were observed for beta-diversity, where data showed a significant time-dependent shift of the microbial profile within both groups (P<0.02, Figure 6 B). Weighted and unweighted UniFrac metrics displays similar bacterial communities in both groups at trial start. Expectedly, community analysis at trial day 28 showed significant differences in composition between groups (P<0.05, Fig. 6 B). This different time-dependent shifts in both groups were even more obvious at end of trial (P<0.01, Fig. 6 B).

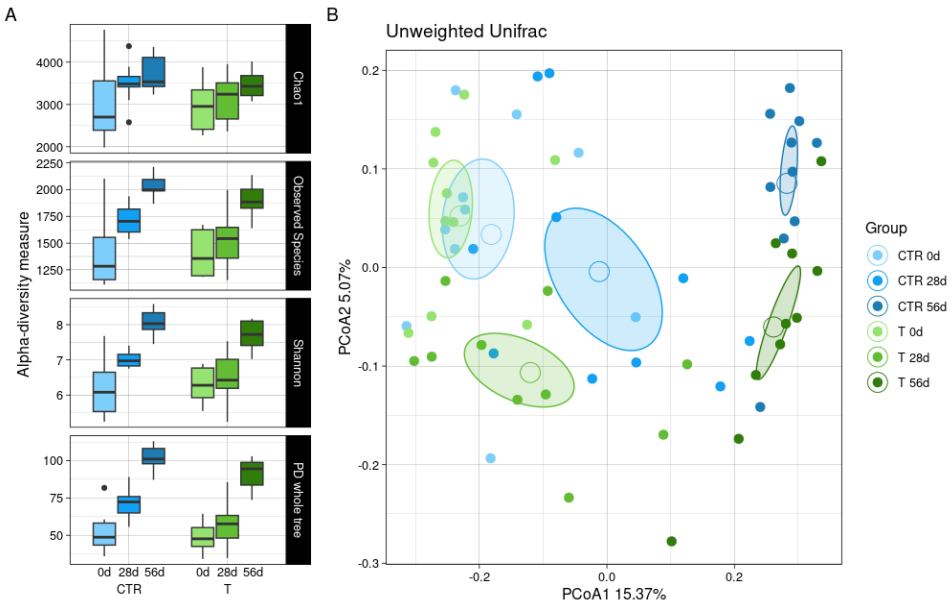

Figure 6. Bacterial alpha (A)- and beta (B)-diversity in fecal samples (n=10 per group) of calves in control (CTR) and probiotic-treated (PRO) group. Analysis is based on 10 samples per group collected at trial day 0, 28 and 56 d.

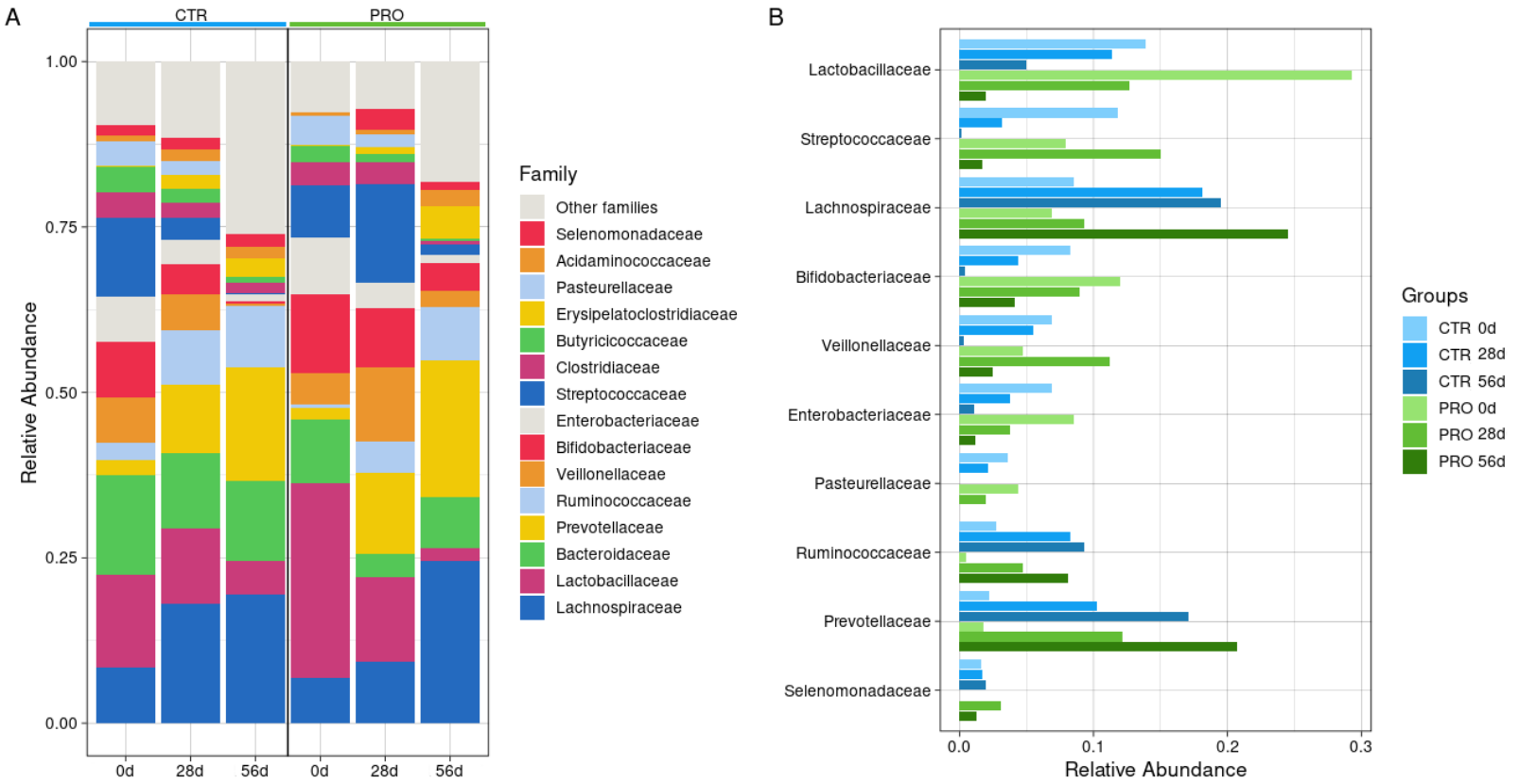

Figure 7. Relative 16S rRNA gene abundances of families in fecal samples of control (CTR) and probiotic-treated (PRO) group. Samples (n=10 per group) were collected on trial day 0, 28, and 56. A: Most abundant families (≥ 1% in at least one sample). B: Selection of families that show significant differences when examining time-dependent and group-specific shifts; the major significant values and comparisons are depicted throughout the text.

Sequence analysis at the family level revealed significant group-specific differences in microbiota composition when examining time-dependent shifts (Figure 7A). Overall, a decreasing trend over time was mainly observed for *Lactobacillaceae, Streptococcaceae, Bifidobacteriaceae, Veillonellaceae, Enterobacteriaceae*, and *Pasteruellaceae*; on the other hand, higher relative abundances characterized the *Lachnospiraceae, Ruminococcaceae,* and *Prevotellaceae* families. (Figure 7B). In particular, the lactic acid-producing family *Lactobacillaceae* was observed to be reduced along the timepoints for both groups, with statistical relevance for PRO (0d *vs* 56d, P<0.001). Similarly, *Streptococcaceae* were also observed to be reduced along time, for both groups (CTR 0d *vs* 56d, P=0.001; PRO 28d *vs* 56d, P=0.0237). An opposite and increasing trend was observed for the butyrate-producing *Lachnospiraceae* family: for the the control group, the comparison between the beginning and the end of the trial reported statistical relevance (P=0.0498 CTR 0d *vs* CTR 56d); for the treated samples, the last timepoint was significantly different from both the start and the mid-point of the trial (PRO 0d *vs* PRO 56d, P<0.001; PRO 28d *vs* PRO 56d, P=0.004).

At the genus level, on the other hand, the relative abundances of *Streptococcus*, *Bifidobacterium, Escherichia-Shigella, Lactobacillus, Ligilactobacillus,* and *Limosilactobacillus* decreased over time (Figure 8A). Notably, significant reductions were observed in (a) both groups for *Bifidobacterium* (CTR: P=0.002; PRO: P<0.001) and *Escherichia-Shigella* (CTR: P=0.002; PRO: P<0.001) and (b) the PRO group for *Streptococcus* (P=0.025) and *Lactobacillus* (P=0.243). Furthermore, the relative abundance in PRO-group samples of *Limosilactobacillus* significantly decreased when comparing samples collected at start and end of the trial (P<0.001) whereas *Ligilactobacillus* significantly decreased between trial day 28 and end of the trial (P=0.008). In contrast, the relative abundances of *Prevotella 9*, *Alloprevotella*, and *Blautia* increased over time in both groups, with a more pronounced increase observed in the PRO group. Specifically, *Prevotella 9* and *Alloprevotella* showed significant increases in PRO-group samples from the start to the end of the trial (*Prevotella 9*: P<0.001; *Alloprevotella* P=0.003), while *Blautia* increased significantly between the start and day 28 (P=0.015), as well as between day 28 and the end of the trial (P<0.001).

Overall, microbiota analysis indicated similar time-dependent shifts in genera across both groups, with varying magnitudes when comparing both groups (Figure 8B). For example, while *Bifidobacterium* decreased in both groups, the reduction was less pronounced in the PRO group compared to the CTR group. Conversely, *Prevotella 9* consistently increased throughout the trial in both groups but to a greater extent in the PRO group compared to the CTR group.

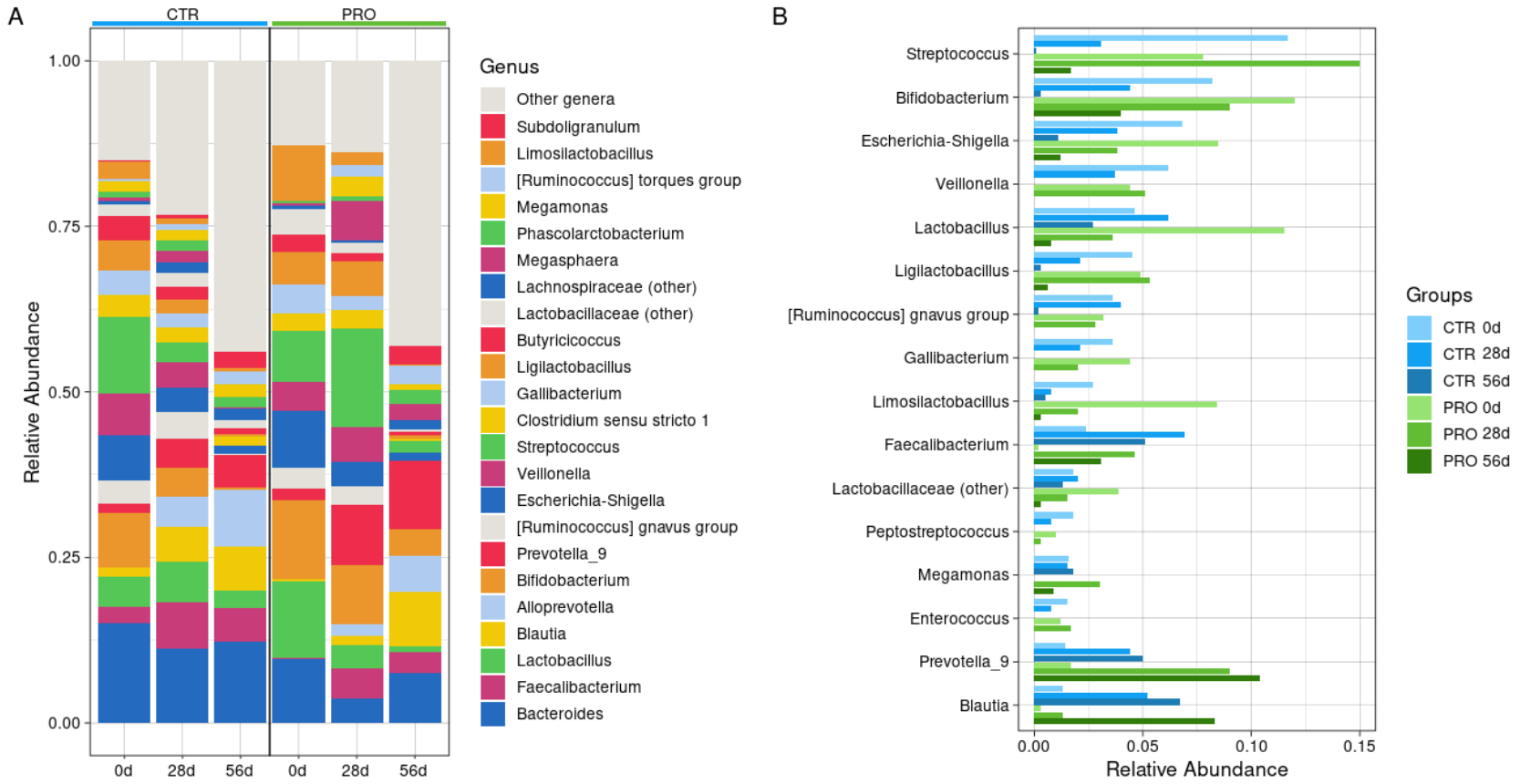

Figure 8. Relative 16S rRNA gene abundances of genera in fecal samples of control (CTR) and probiotic-treated (PRO) group. Samples (n=10 per group) were collected on trial day 0, 28, and 56.A: Most abundant genera (genera with ≥ 1% in at least one sample). B: Genera that show significant differences when examining time-dependent and group-specific shifts

Functional microbial metabolic prediction and correlation with detected taxa

Functional microbial metabolic prediction is reported in figure 9 (A and B). No interaction between treatment and time effect (i.e. CTR vs T at the same time-point) was depicted in conditioning functional metabolic prediction. However, when analyzing changes over time within the two groups, the main differences were observed between 0 and 56 d sampling time points for both CTR and T. CALVIN-PWY (Calvin–Benson–Bassham cycle) prediction was significantly enriched in both group at 56 d compared to 0 d ($P<0.05$). Furthermore, while ANAGLYCOLYSIS-PWY functional prediction was found enhanced in CTR at 56 d compared to 0 d, a reduction at 28 d was detected in T group in comparison to 0 d. On the other hand, pyruvate fermentation to isobutanol (PWY-7111) was found increased in CTR and T group from 0 to 56 d. In CTR group, uridine monophosphate (UMP) biosynthesis (PWY-5686) increased from 0 to 56 d ($P<0.05$). Finally, the pentose phosphate pathway (NONOXIPENT-PWY, non-oxidative branch) predicted abundance was found increased in T group at 56 d in comparison to 0 d ($P<0.05$). Correlation analyses at genus level revealed significative association among specific taxa with detected MetaCyc pathways as displayed in figure 8 C. In particular, CALVIN-PWY was negatively correlated with *Veillonella*, *Ruminococcus gnavus* group, *Gallibacterium*, *Escherichia-Shigella*, *Clostridium sensu stricto* 1, *Streptococcus*, *Bifidobacterium*, *Lactobacillus* and *Limosilactobacillus* ($P<0.05$). Nevertheless, the same pathway was positively correlated with *Faecalibacterium*, *Blautia*, *Prevotella 9* and *Alloprevotella* ($P<0.05$). *Alloprevotella* and *Blautia* were also positively correlated with ANAGLYCOLYSIS-PWY as in the case of *Lactobacillus* and *Limosilactobacillus* ($P<0.05$). However, the same pathway was negatively related to *Veillonella*, *Ruminococcus gnavus* group, *Gallibacterium*, *Escherichia-Shigella*, *Clostridium sensu stricto* 1 and *Streptococcus* ($P<0.05$). Moreover, UMP biosynthesis pathway (5686-PWY) reflected a similar trend in terms of correlation as depicted by Fig. 8 C. In addition, NONOXIPENT-PWY was positively correlated with *Streptococcus* and *Blautia* but negatively with *Bacteroides*, *Gallibacterium*, *Escherichia-Shigella* and *Limosilactobacillus*.

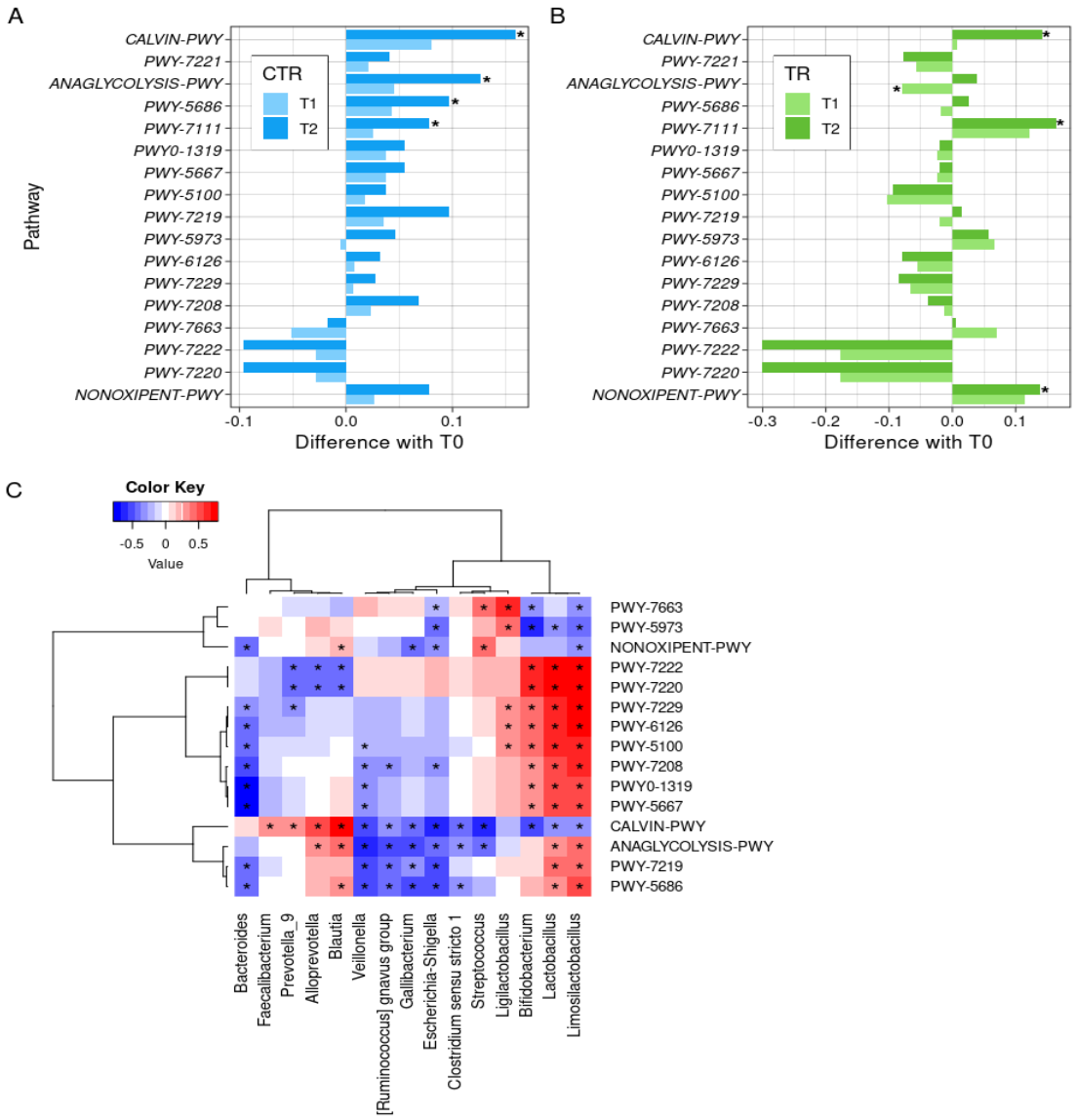

Figure 9. Functional microbial metabolic prediction performed in CTR and T group (A and B respectively) following bioinformatic processing (n=10 per group). Bacterial taxa correlations with MetaCyc pathways are displayed (C). The colours of the squares indicate the negative (blue) and positive (red) correlation (*=P<0.05).

Discussion

Considering the presence of different etiological agents, diarrhea occurring within the first days of life represents one of the most concerning causes of mortality, morbidity and economic loss in calves rearing (Brunauer et al. 2021; Cho and Yoon, 2014). Moreover, during the first hours of life, quality of colostrum is pivotal for an efficient passive immunity transfer, for the onset of a balanced gut microbiota and to prevent enteric diseases which can further affect animals performances and productivity (Gomez and Chamorro 2017). However, the combination of a failure in passive immunity transfer and the proliferation of diverse entero-pathogens could exacerbate the outburst of diarrhea phenomena in herds of calves. Therefore, nutritional interventions enable to ensure the establishment of a balanced gut environment and avoiding a wider antimicrobial usage became pivotal (Savoini et al. 2010). The administration of probiotics became a widespread practice to improve health of pre-weaning calves (Agazzi et al. 2014). As live microorganisms, their strain-specific mechanisms of action are highly divers and include (a) (a) competitive exclusion of potential harmful bacteria, (b) improvement feed conversion by digestive enzyme production, (c) direct inhibition of potentially harmful bacteria due to the production of antimicrobial substances, (d) production of beneficial short chain fatty acid and vitamins, and (e) immune modulation as well as anti-inflammatory action (Alagawany et al. 2018; Raheem et al. 2021). Despite the presence of a wide range of probiotics strains available on the market, the selection of newly strains capable to guarantee technological advantages is strongly needed. *Lactobacillus* and *Bifidobacterium* represents the most common choices in terms of nutritional application of probiotics as reported by Zhou et al. (2020). Nevertheless, the author indicate that a good part of these strains is not resistance to digestive conditions. Indeed, despite their potential in ameliorating gut conditions, *Lactobacillus* and *Bifidobacterium* species can be characterized by a low survival rate ranging from 1 to 15% (Keller et al. 2019). Therefore, the research on strains able to form endospores and, therefore, resist in the digestive tract became increasingly important. Specifically, strains of *Bacillus and Weizmannia* are characterized by the capacity to generate endospores able to efficiently survive in the gastrointestinal tract, promoting digestive enzymes activities and modulating gut microbiota composition (Cao et al. 2020). In the present study, the administration of a specific *Weizmannia* strain was considered. Zhang et al. (2021) underlined the capacity of *Weizmannia* (5×10^9 CFU/kg of complete feed) to improve the growth performance of broiler chickens. Authors reported that the dietary supplementation with *Weizmannia coagulans* enhanced BW of chickens at 21 and 42 d after hatching, maintaining a better ADG all over the trial but without influencing ADFI or feed conversion. In addition, 400 mg/kg feed of *W. coagulans* (>5×10^9 CFU/kg of complete feed) improved the ADG and the ADFI of weaning piglets in a 28-day trial (Fu et al. 2021). Frizzo et al. (2010) reported that the administration of an orally administrated inoculum of bovine origin composed of *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T, improved body weight gain and final body weight of Holstein calves in a 35-day trial in which calves were fed a milk replacer supplemented with spry-dried whey powder. More recently, an administration of

Lactobacillus plantarum 299v in milk replacer (1×10^{10} CFU/ml) improved the ADG of Holstein calves (Jiang et al. 2020). Even though the potential of probiotics in ameliorating calves growth performance (body weight gain and dry matter intake) is widely recognized during the first 60 days of life (Frizzo et al. 2011) data specifically referring to *Weizmannia* are still lacking in available literature. However, in our study better final BW and ADG were registered in the PRO group. These data are in accordance with what was previously discussed, outlining the capacity of the tested strain in improving the growth of young female Holstein calves during the weaning transition. In our study, body morphological measurements were not conditioned by the treatment. Our results are in line with previous observations in which the administration of *Lactobacillus casei*, *Lactobacillus salivarius* and *Lactobacillus sakei* (1:1:1) at 1×10^{11} CFU/g did not affect hearth girth and hip height changes when of Polish Holstein calves in a 56-day trial (Stefańska et al. 2021). In contrast, Agazzi et al. (2014) highlighted that a mixture of *Lactobacillus animalis* SB310*, Lactobacillus paracasei* subsp. *paracasei* SB137 and *W. coagulans* SB117 in a 30:35:35 ratio (1.8×10^{10} CFU/g, 1 g/day per calf) enhanced the heart girth of Holstein calves at 28 d of life. In our study, despite a strong tendence towards an enhanced withers height in the treated group very similar values among groups considering all the evaluated biometric parameters were registered. In addition, the potential of *Weizmannia* supplementation in ameliorating nutrient digestibility following an increase in endogenous digestive enzymes secretion has been previously discussed (Gu et al. 2015; Zhao et al. 2018). This capability may support also the development of a small intestine tissue increasingly able to digest and absorb complex nutrients. In addition, *Bacillus* spp. supplementation is recognized in efficiently shaping the lipid metabolism and storage in different animal models (Lee et al. 2022; Wu et al. 2018). Therefore, it is reasonable to sustain the hypothesis that the better growth performances registered in the PRO group were driven by body fat deposition rather than morphological frame development (Casper et al. 2021).

Diarrhea occurrence may reach a peak from 7 to 14 days from birth (Cho and Yoon, 2014; Araujo et al, 2015). In addition, calves are characterized by a diarrheal status when considering a fecal score above 1 in a four-points (0 to 3) fecal score evaluation (Santos et al, 2015; Gomez et al, 2017). In our study, considering the first 7 days of trial (7 to 14 from birth) CTR calves were characterized by a persistent diarrheal status (fecal score >1) until day 6 on trial, whereas PRO animals reached a normal fecal consistency (below 1) after day 3 on trial. The supplementation *Weizmannia* revealed positive effects on the fecal consistency of piglets (Sun et al. 2022). Moreover, as reported by the previously discussed study performed by Agazzi et al. (2014) the administration of a probiotic mixture containing *W. coagulans* SB117 reduced the incidence of diarrhea at 28 d in Holstein female calves. Furthermore, Zhang et al. (2019) highlighted the capacity of *Lactobacillus rhamnosus* GG (1×10^{10} CFU/d) in ameliorating the fecal score of male Holstein calves during a 6-week trial.

Similarly, in the present study results outlined an improved fecal scoreof the probiotic-treated group during the first two weeks of trial (from day 7 - 21 of life), underlining the capacity of the tested

Weizmannia strain DSM 32016 to ameliorate fecal score of female Holstein calves during early phases of the weaning transition.

Bayatkouhsar et al. (2013) evaluated a commercially available probiotic composed of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidium* and *Enterococcus faecium* and a laboratory multi-strain probiotic containing *Lactobacillus acidophilus* PTCC 1643, *Lactobacillus rhamnosus* PTCC 1637, *Lactobacillus casei* PTCC 1608 and *Lactobacillus delbrueckii* PTCC 1333 (both supplemented at a total concentration of 2.0×10^8 CFU, 2g/d/calf). The authors reported a lack of effect on hematocrit and WBC in blood samples collected 7 days prior the end of the weaning period, and 24 h and 7 days post weaning. Furthermore, from day 7 to 90 of life plasma BHB, NEFA and glucose were not conditioned by the two probiotic treatments. Similarly, Wang et al. (2022) found that the administration of 1.2 g/d per calf of a compound probiotic consisting of *Lactobacillus plantarum* (10^8 CFU/g), *Pediococcus acidilactici* (10^8 CFU/g), *Pediococcus pentosaceus* (10^8 CFU/g), and *Bacillus subtilis* (10^7 CFU/g) in milk replacer did not affect the glucose plasma level of Holstein calves at 40 and 80 d of life. Our results are in line with the discussed literature, as BHB, NEFA and glucose were not modulated by the probiotic treatment in this study. Probiotic *Bacillus* and *Weizmannia* strains are known for their capacity to exert an immunomodulatory effect on the host, which is mainly due to the activation of dendritic cells and macrophages that can result in an enhanced production of secretory IgA and different other immune-associated molecules such as cytokines (Luise et al. 2022). Furthermore, probiotics can stimulate B cell proliferation in Peyer's patches resulting in an enhanced IgG secretion in blood (Rousseaux et al. 2023). Sun et al. (2010) observed an increase in IgG plasma levels when directly feeding *Bacillus subtilis natto* (1×10^{10} CFU) to Holstein bull calves at the end of the weaning transition (day 51 of life). In addition, Wu et al. (2021) found that the administration of a multi-strain probiotic composed of *Lactobacillus acidophilus* S5, *Bacillus subtilis* No. Bzg988118, and *Saccharomyces cerevisiae* SHZ2017 (2 g/d per calf) positively affected the level of IgG in plasma of Chinese Holstein female calves between week 4 and 8 of life but without reaching significance when considering the time*treatment interaction. In our study, treated calves reached a higher concentration of plasma IgG at day 56 of trial, corresponding to day 63 of life. These results are in line with the discussed data and testified the capacity of *WF-32016* to improve the immune status of female Holstein calves. Probiotics antioxidant capacity is linked to enhanced production of superoxide-dismutase (SOD), catalase (CAT), metal chelation and scavenging of reactive oxygen species (ROS) but also to a partial reconstitution of the host intestinal microbiota composition during challenging phases (Feng and Wang, 2020). Indeed, it is well known how lactic acid bacteria can positively influence the redox balance (Bryukhanov et al. 2020). Enhanced total SOD and CAT were found in Murrah buffalo calves supplemented with a compound probiotic (*Lactobacillus reuteri* BF-E7 and *Lactobacillus salivarius* BF-17; 1 g/ d per calf) at 30 d of life (Varada et al. 2022). Moreover, a supplementation of 500 mg/d of *Bacillus megaterium* (10^{10} CFU/g) enhanced glutathione (GSH) at 14 d of life and decreased malondialdehyde (MDA) in serum of Holstein calves. However, serum SOD was also increased in

Holstein calves at day 80 of life by administering a multi-strain probiotic containing 10^7 CFU/g of *Bacillus subtilis* (Wang et al. 2022) but lacking an effect on the total antioxidant capacity (T-AOC). In contrast, in our study serum T-AOC of female Holstein calves was positively conditioned at the end of the trial (day 56) by supplementing 10^9 CFU/kg of *WF-32016* in starter feed and CMR. In this case, the collected results reinforced the potential of this lactic acid-producing strain to protect the host towards oxidative stress.

The establishment of a complex and diversified microbial community in the gut environment is crucial for the host to avoid further detrimental dysbiosis (Trevisi et al. 2018). In a previous study performed on broilers, the administration of 200 mg/kg of *W. coagulans* TBC169 (1×10^9 cfu/kg) revealed limited effects in modulating Simpson and Shannon indexes of microbial diversity in jejunal microbiota after 21 days and 42 days of administration (Li et al. 2019). On the contrary, Sun et al. (2022) evaluated the administration of 600 mg/kg of *W. coagulans* in weaning piglets, finding marked modulation of fecal Shannon and Chao1 indexes both at phylotype and family level in comparison to a control group at day 21 after weaning. Notably, the authors reported that after 28 days of administration, the *Weizmannia*-fed group showed significantly different genus level similarities in β-diversity evaluation in comparison to the control group. Zhang et al. (2021) evidenced comparable results as the administration of 5×10^9 CFU/kg of *Weizmannia* did not influence α-diversity indexes at day 21 and day 42 after hatching in cecal content samples. However, authors reported significant changes in principal component and coordinate analyses, as the Weizmannia-fed group showed appreciable differences if compared to a control and an antibiotic treated group (75 mg/kg of chlortetracycline) despite marked interindividual variation.

Our results are in line with the literature as alpha diversity indexes were not conditioned by the treatment. Nevertheless, after trial day 28 of administration weighted and unweighted UniFrac metrics revealed marked differences between CTR and PRO, which were confirmed at end of trial, underlining the potential of *WF-32016* administration to drive significative changes in fecal microbiota composition over time. At family level, the increased relative abundance of *Bifidobacteriaceae*, *Lachnospiraceae*, and *Prevotellaceae* in the PRO group, particularly at the end of the trial compared to the CTR group, may collectively contribute to the improved fecal consistency observed in this study. *Bifidobacteriaceae* are associated with enhanced fibre digestion and microbial cross-feeding, which can stimulate additional butyrate production (Rivière et al., 2016; Wang et al., 2022). Recent studies indicate that butyrate is a vital regulator of epithelial cell proliferation, differentiation, and apoptosis in the stomach and small intestine of calves and piglets, thereby stimulating rumen development and positively affecting the growth and health of calves (Górka et al., 2011). In fact, further studies have shown that increased abundance of *Bifidobacteriaceae* is positively correlated with early-life growth in calves (Zhuang et al., 2024). Members of *Lachnospiraceae* are known to utilize intestinal lactic acid to produce butyric acid

(Duncan et al., 2004; Sikora et al., 2013), supporting the theory that lactic acid produced by WF-32016 is utilized by other health-promoting bacteria, particularly butyrate producers. Members of *Prevotellaceae* play a key role in producing short-chain fatty acids and amino acids in the rumen. Its abundance is influenced by dietary fibre levels, making it especially relevant during calf weaning when fibre-rich feeds like hay and calf starter replace milk (Beaver et al., 2021).

At genus level, the increased abundance of *Prevotella 9*, especially at the end of the trial in the probiotic-fed group compared to the control group, suggests an increase in bacteria known for complex carbohydrate and protein digestion, propionate production, and amylolytic properties (Betancur-Murillo et al., 2022). This is particularly important for young calves, as their ability to efficiently digest starch is limited during the weaning transition (Dias et al. 2018). However, an increase of such propionate producers has also the potential reduce methane production in older animals (Betancur-Murillo et al., 2022). Ruvalcaba-Gómez et al. (2023) evaluated the administration of a *lactic acid-producing probiotics* to dairy calves (1×10^9 CFU/kg weight) resulting, as in this study, in an increase of *Prevotellaceae*. Furthermore, the authors linked *Prevotella* and *Alloprevotella* genera to a better fecal score in calves treated with probiotics. In addition, *Blautia* relative abundance was found increased following the administration of a compound probiotic (*Lactococcus lactis*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum*) along with compound yeast (*Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) at 15 d in dairy calves. In this specific case, the author reported the anti-inflammatory potential of this genus. Moreover, in the present study *Blautia* genus showed a stronger increase over time in the PRO group than in the CTR group. *Blautia* has been positively correlated to increased starter intake and enhanced endogenous short-chain fatty acids (SCFAs) production in the gut of young dairy calves (Lu et al. 2022). *Blautia* was appointed as a potential biomarker of intestinal health, most likely due to health-beneficial butyrate production (Benítez-Páez et al., 2020). The time-related increase of *Blautia* especially in WF-32016-fed calves most likely contributed to better fecal consistency observed during the trial in the PRO group (Li et al. 2022). The decrease of relative abundances of *Streptococcus*, *Lactobacillus* and *Bifidobacterium* throughout the trial is following the common intestinal microbiota development in dairy calves (Du et al. 2023). Shifts in *Streptococcus* were similar in both groups, whereas differences in relative abundances of *Lactobacillus* within the probiotic group were not affected by treatment, time, or their interaction which was most likely based on high inter-variability among groups at trial start. Nevertheless, the relative abundance of *Bifidobacterium* was constantly higher in fecal samples of the PRO group throughout the trial. *Bifidobacterium* can be related to better exogenous carbohydrates utilization as well as acetate and lactate production which can positively influence calf growth (Zhuang et al. 2024).

Analysis of predicted bacterial pathways indicated, a clear increase of relative abundance of sequences associated to the non-oxidative branch of pentose phosphate pathway in the PRO group, whereas sequences related to orthologs for glycolysis were increased in abundance in the CRT group. The non-

oxidative branch of pentose phosphate super-pathway influences diversified metabolic processes associated with amino acids biosynthesis and is crucial for glycolysis (Rashida and Laxman, 2021). Therefore, the observed time-related increase in abundance of the pentose phosphate pathway within PRO-group samples may indicate a higher energy turnover utilized for biosynthesis related to body function maintenance, which has been previously linked to better growth performance (Peng et al. 2024). Considering the overall fecal microbiota analysis, the number of bacterial genera observed seems to demonstrate differential longitudinal changes over time, with CTR and PRO groups showing greater differences after extended probiotic administration of WF-32016. As most of the treatment effects were observed at the end of the trial and considering the rapid turnover of *Bacillus coagulans* spores (Abhari et al. 2015), it is noteworthy that it is well accepted that some effects of *probiotics* depend on a constant administration, which then can further exert positive reflexes during the weaning transition of calves.

Conclusions

The collective results of this study demonstrate that probiotic *Weizmannia faecalis* DSM 32016 (formerly *Bacillus coagulans*) administered to Holstein calves improved growth performance, fecal score, immunity, antioxidant status and intestinal microbiota composition. Furthermore, the data indicate a potential probiotic-driven modulation of super-pathways related to glycolysis and biosynthesis processes. Overall, this study highlights the positive effects of *Weizmannia faecalis* DSM 32016 intestinal and overall health in Holstein calves.

Ethical Statement

The practices conducted during the trial were evaluated and approved by the University of Milan Animal Welfare Committee (OPBA_32_2023, 24/02/2023).

Disclosure statement

No potential conflict of interest was reported by the authors

Data availability statement

Data are available from the authors upon reasonable request from the corresponding author: raffaella.rebucci@unimi.it

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CHAPTER 9: Prevention of *Escherichia coli* induced post-weaning diarrhea by parenteral vaccine stimulation of secretory IgA

Brief introduction to the study: piglets' post-weaning diarrhea prevention represents a pillar aim to minimize the spread of antimicrobial resistance (AMR). During my PhD, I had the opportunity to investigate alternative strategies beyond nutritional approaches and based on innovative vaccination protocols against *E. coli*. In particular, the following pages will be dedicated to a brief report derived from a 6-month secondment at the Department of Animal and Veterinary Sciences of Aarhus University (Denmark) where the Gut and Host Health group kindly hosted me. During this period, I had the possibility to evaluate the efficacy of an immunization protocol based on a bivalent vaccine supported by Cationic Adjuvant Formulation (CAF) liposomes applied in suckling piglets. After weaning, piglets were challenged with *Escherichia coli* expressing F4 and F18 adhesion virulence factors to further investigate the immunization capacity of the vaccine. Therefore, part of my activity involved the analyses of fecal samples to evaluate F4/F18 shedding. As the project is ongoing, in the present report a background section will introduce the study and will be followed by a brief description of the analyses performed and part of the preliminary data collected during these 6 months experience.

Introduction

Post-weaning diarrhea (PWD) still represents one of the major concerns in swine industry. The outburst of diarrheal disorders during weaning is caused by *E. coli* enterotoxigenic strains (ETEC). Escherichia coli is an ubiquitarian potential pathogen widely present in the small intestine of piglets after birth (Jacobson, 2022). Following weaning, the typical transient anorexia is accompanied by intestinal tissue regression, enteric and systemic inflammatory responses, enhanced intestinal permeability and loss of gut barrier integrity (Blavi et al, 2021; Lallès and Montoya, 2021). These dynamic changes are characterized by a loss of bacterial diversity which furtherly decrease commensals competitiveness towards opportunistic microorganisms (Kim et al, 2022). As a consequence, ETEC strains are facilitated in proliferation process. ETEC strains are able to adhere the enteric mucosal surface through F4 and F18 virulence factors (Rhouma et al, 2017). In addition, ETEC can produce heat-labile (LT) and/or heat stable (STa, STb) toxins which are associated to liquid and electrolytes accumulation and further intestinal impairments (Luppi et al, 2016; Matsumoto et al, 2020). Secretory IgA are the first defense tool to counterattack ETEC infection (Riaz et al, 2020). The gut mucosal immune system is composed by a wider ensemble of lymphocytes and antibodies in the animal organism. B-cells within Peyer's patches layer play a key role in regulating secretory IgA production once activated (Corthesy, 2013). Furthermore, in gut associated lymphoid tissue (GALT), T-cells regulate secretory IgA production through Th1 and Th2 cytokines which, respectively, enhance and decrease mucosal IgA secretion. Therefore, it is reasonable to consider that a balanced activity among Th1 and Th2 cells is favorable to a normal secretory IgA production (de Groot et al, 2021). In addition, Th17 have been pointed out as the primary cells group in secretory IgA regulation (Cao et al, 2012). To prevent ETEC infections and PWD while favoring gut development and local immune response of weanling piglets, both antibiotics and pharmacological dosages (2500-3000 ppm) of zinc oxide (ZnO) orally administered have been extensively adopted for many years. However, public health and environmental issues related to antimicrobial resistance spread and heavy metal compounds accumulation in European soils brought to the ban of growth-promoters antibiotics and pharmacological ZnO (Bonetti et al, 2021). Consequently, the necessity to search alternative strategies able to positively condition piglet intestinal health emerged. Modulating the adaptive response of the immune system towards ETEC through vaccination has been indicated as a particularly effective strategy (Zhang et al, 2018). However, vaccination efficacy is based on the expression of specific antigens by ETEC, and in most cases, F4 and F18 fimbriae are considered in vaccines development to inhibit ETEC adhesion as starting event of PWD. Nonetheless, the wide variety of adhesion and virulence factors can represent an obstacle which strongly conditions vaccination efficacy (Ramis et al, 2021). To overcome such an issue, in recent years vaccine development focused on the possibility to obtain

immunization towards both adhesion factors and toxins as commercially available vaccines are not sufficiently polyvalent (Dubreuil, 2020). In addition, both parenteral and oral monovalent or bivalent solutions based on inactivated *E. coli* are available on the market. Oral vaccination has been highlighted for notable advantages related to costs of realization, facilitated large-scale administration, safety and stability along with the possibility to minimize stressful events for the animal (Ascón et al, 1998; Kotton et al, 2004). Furthermore, antigens oral administration may represent the preferential route to specifically targeting the gut tissue immune response through specific secretory IgAs production. Indeed, ensuring protection against *E. coli* further requires the presence of previously stimulated memory B cells. However, oral vaccination may exert limitations related to gastro-enteric environmental conditions and lactogenic immunization when applied in suckling piglets, whereas encapsulation technologies may fail in protecting antigens (Van damme et al, 2011). On the other hand, parenteral vaccine injections have been linked to systemic IgA conditioning rather than local immune response modulation. In the case of parenteral administration, even though secretory IgA production maintenance is possible through systemic IgA promotion, memory response in GALT is lacking (Melkebeek et al, 2013). Therefore, to trigger a strong mucosal response through Th17 activation following parenteral injections, Cationic Adjuvant Formulation (CAF) platforms based on liposomes were developed (Rosenkrands et al, 2005). Supplementing parenteral vaccines with retinoic acid increased intestinal secretory IgA response in mice (Tan et al, 2011). From these findings, Christensen et al. (2019) developed new generation adjuvant based on CAF platform including retinoic acid (CAF23b) characterized by specific immunization kinetic. Evaluating the adjuvant efficacy on mice, the author showed to favor an isotype shift of intestine-homing B cells into SIgA-producing plasma cells by retinoic acid addition. Altogether, these considerations brought to the possibility of formulating new functional vaccines to stimulate mucosal immune response in post-weanling piglets. Therefore, the aim of the present study is to evaluate the effects of a bivalent vaccine (F4/F18 fimbriae) supported by CAF platform during an immunization protocol for suckling piglets to further stimulate intestinal secretory IgA response following an induced ETEC F4 and F18 challenge.

Material and methods

Ethical statement

The study was performed at the facilities of the Department of Animal and Veterinary Sciences of Aarhus University (Denmark). The animal experimental procedures were carried out in compliance Danish Ministry of Justice Law no. 474/15.05.2014 concerning animal experiments and care. Therefore, permission to perform the present study was given by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, the Danish Veterinary and Food Administration.

Animal housing and experimental design

The study comprised litters of 10-12 piglets from six sows (Duroc x Landrace x Yorkshire mated with Norsvin Landrace boar) conducted in two consecutive rounds. Sows have been tested for sensitiveness towards F4 and F18 and confirmed homozygote carriers of the dominant genes encoding ETEC F4 (MUC4 gene) and F18 ($FUT1^{GG}$ gene) fimbriae receptors (Jenkins et al, 2024; Jerez-Bogota et al, 2023). Therefore, piglets were confirmed susceptible to ETEC F4 and F18. Litters were equalized on day 2. Newborn piglets were housed 3.0×2.2m farrowing crates pens for sows (Skiold Jyden A/S, DK). Supplemental heating was provided through dedicated bulbs (150 W) until day 7-10 of life. Suckling piglets received exclusively sow's milk until day 10 of life. Creep feed was administered to piglets from 10 to 25 of life and a starter diet followed until weaning. For each litter, half of the piglets were vaccinated on day ~3 after birth. Afterwards, vaccination booster was administered 10 days before weaning. Non-vaccinated piglets received a placebo treatment following the same timeline protocol. After weaning (age 27-30 d), a total of 77 mixed sex piglets (40 piglets round 1, 37 piglets round 2) were divided into 4 experimental groups. The experimental groups were organized as follows: Placebo non-challenged (P-nonCh; 4 piglets per round), Placebo challenge (PCh; 16 piglets in round 1 and 15 in round 2), Vaccine non-challenge (V-nonCh; 4 piglets per round) and Vaccine challenge (VCh; 16 piglets in round 1 and 14 in round 2). During round 2, 3 piglets (1 PCh and 2 VCh) were excluded from the trial due to hoof abscesses. After weaning, littermates were housed for 21 days in 2.15 x 1.10 m pens of challenged or non-challenged pairs. Pens had slatted and heated floor and physical contact between different pens was avoided to limit cross-contamination. Piglets were allocated in a room where temperature was gradually regulated from 25 °C until reaching ~21 °C at the end of the third week. Relative humidity was maintained approximately at 62%. Weaning piglets were fed ad libitum with the same basal diet supplied from day 25 of life. Starter diet was formulated to satisfy the Danish nutrient requirement standards of pigs (Tybirk et al, 2015).

F4 and F18 Enterotoxigenic *Escherichia coli* challenge protocol

Challenge with F4 and F18 ETEC strains was applied following the procedures described by Jerez-Bogota et al. (2023) and Hansen et al. (2024). *Escherichia coli* serotype O149 F4 (ETEC 9910045-1) expressing F4 fimbriae (virotype F4ac), heat-stable enterotoxin (STb), heat-labile enterotoxin (LT) and enteroaggregative E. coli heat-stable enterotoxin 1 (EAST1); and serotype O138 F18 (ETEC 9910297-2STM) expressing STb, LT, EAST1 and Shiga toxin type 2e (Stx2e) were isolated from piglets manifesting diarrheal disorders and furnished by the Danish Veterinary Institute (Copenhagen, Denmark). ETEC F4 and F18 were found to be hemolytic after blood agar culture. Afterwards, colonies of both serotypes were transferred to BHI broth and incubated. Concentrations of bacterial suspension were confirmed by plate count on blood agar. The pigs received 5 ml of a solution containing $1x10^9$ cfu ETEC F4 and F18 in block 1 and $2x10^9$ cfu ETEC F4 and F18 in block 2 on day 1, 2 and 3 post-weaning. Placebo piglets received 5 ml of a saline solution (0.9% NaCl). A polyethylene tube connected to a syringe (6 ml of volume) was used to facilitate the oral administration of the solutions described.

ETEC counts and Quantitative Polymerase Chain Reaction

Fecal samples were collected during the first week post-weaning (1-7 days) and on day 9, 11, 14, 17 and 21 after weaning. Quantitative polymerase chain reaction (qPCR) was used for quantification of the gene encoding the F18 (*fed*A gene) and F4ac fimbriae (*fae*G) in collected samples. NucleoSpin 96 DNA Stool kit (Macherey-Nagel, Düren, Germany) was used to extract DNA from 50 mg of fecal. Genomic dsDNA was assessed with Qubit Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supported by an Invitrogen Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). qPCR was performed on a ViiA 7 real-time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) using a MicroAmp Optical 384 well reaction plate (Applied Biosystems, Waltham, Massachusetts, USA). The qPCR reactions contained 5 µL of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), the F18 and F4 primers at a concentration of 0.3 mM, 2 µL of template DNA and water to a final volume of 10 µL. Analytical triplicates were considered for all samples and the data were elaborated in QuantStudio real-time PCR Software v1·4. Ct cut-off values were 31.3 for F4 and 31.0 for F18. Lower limit of quantification (LLoQ) of 5 and 4 copies were identified for F4 and F18, respectively. Sequence of primers and qPCR settings are represented in table 1.

Table 1. Sequence of primers with relative qPCR settings and references.

Primer name	**Target sequence**	**Sequence (5'-3')**	**Conc[1] (mM)**	**T_A[2] (°C)**	**Size[3] (bp)**	**Reference**
F18 F/R	*E. coli* F18 fimbriae (*fed*A)	GGAGGTTAAGGCGTCGAATAG CCACCTTTCAGTTGAGCAGTA	0.3	65	86	Frydendahl et al, 2001
F4 F/R	*E. coli* F4ac fimbriae (*fae*G)	CACTGGCAATTGCTGCATCT ACCACCGATATCGACCGAAC	0.3	62	90	Wang et al, 2017

[1]Concentration in qPCR reactions

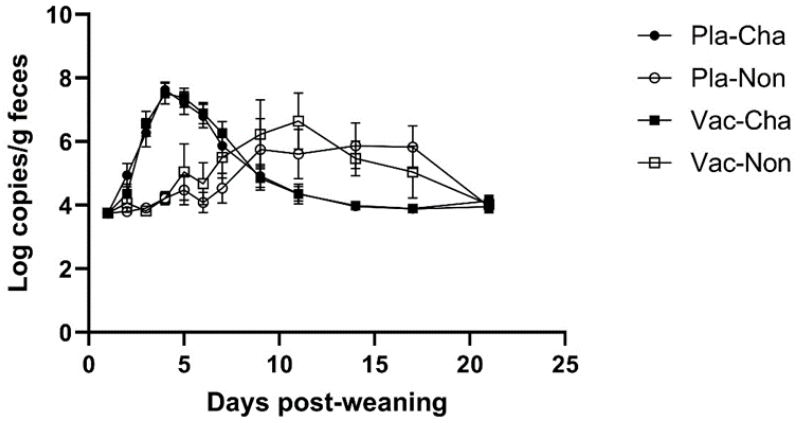
[2]Annealing temperature

[3]Amplicon size

Preliminary Results

Data collected from F4 and F18 shedding evaluation following qPCR analyses are reported in figure 1. Data were elaborated on SAS Studio Version 9.2 (SAS Institute Inc., Cary, NC, USA). Data sets are represented considering the overall results (both rounds). Data are represented as log copies/g of sample ± SEM.

A



B

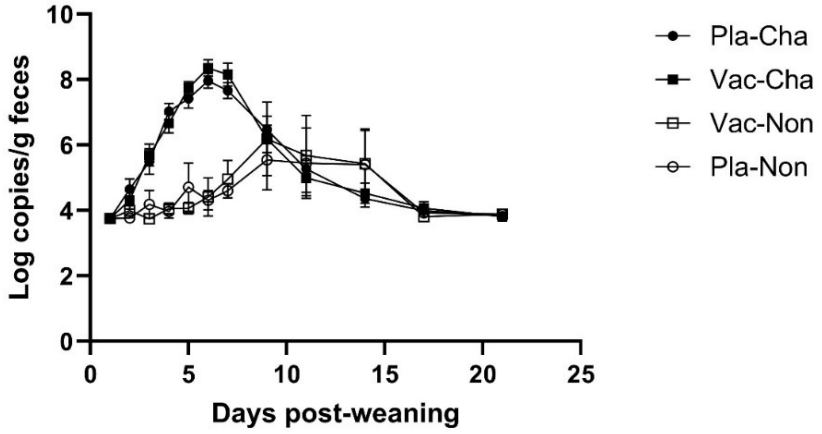


Figure 1. Effect of ETEC F4 and F18 challenge and vaccination on shedding of A) F4 fimbria (*FaeG gene*) and B) F18 fimbria (*fedA* gene) in feces. The ETEC F4 and F18 was orally administered on day 1, 2, and 3 post-weaning. Data are presented as means ± SEM. Pla-Cha: placebo, challenged, n=23; Pla-Non: placebo, non-challenge, n=6; Vac-Cha: vaccine, challenged, n=20; Vac-Non: vaccine, non-challenge, n=6.

Concluding remarks

Preliminary data from this study did not show an effect on F4 and F18 shedding by the vaccine administration. In particular, Pl-Cha and Vac-Cha followed similar trends evidencing a lack of appreciable differences. Blood samples were collected on weaning day and seven days post-weaning for acute phase protein concentration analysis. Further, data on DM determination of fecal samples and growth performance will be done to conclude on the tested vaccine efficacy. Nonetheless, from a personal point of view, this experience was extremely formative and helpful in consolidating my knowledge about bacterial challenges trials and laboratory practices, especially when dealing with *E. coli* virulence factors shedding evaluation.

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CHAPTER 10: General discussion and conclusions

The livestock sector is facing a wide range of challenges which involve the continuous research and development of more effective strategies in sustaining animals and public health along with environmental pollution mitigation. Negative events such as wars and pandemic deeply affected livestock sector productive capacities and economy. In this context, European institutions are regularly adopting and updating policies to accentuate the need for more sustainable strategies without excluding animals and humans welfare and safety. Therefore, a One-health approach striving to minimize antibiotic usage and AMR profusion is strictly needed. From this point of view livestock nutrition and the application of feed additives in particular, assume a primary role. Both in young monogastrics and ruminants, the weaning transition represents one of the most critical phases in which animals are particularly susceptible to enteric diseases. To solve them, antibiotics are still the main compounds supplied in feeds, especially in developing countries. On the other hand, the past extensive use of high dosages of trace elements to prevent diarrheal disorders in weanling piglets contributed to AMR spread worldwide. My research project focused on alternative strategies based on feed additives to increase the gut health of monogastrics and ruminants during the weaning transition. Weanling piglets and pre-weaning dairy calves display similarities from a physiological point of view during this critical phase. In particular, common traits can be found in gut microbial niches development and functions, intestinal tissue immune response and gut barrier integrity development. Moreover, for both species diarrheal disorders caused by enterotoxigenic *E. coli* strains still represent a major cause of mortality, morbidity and economic loss. The present project not only focused on the administration of additive compounds enabled to exert multiple effects on gut health, but also evaluated the optimization of feed additives supplementation thorough their synergisms. During the first trial, weanling piglets raised in a local commercial farm were fed a basal diet supplemented with a blend of carvacrol, tannic acid and glycerides of MCFAs (capric, caprylic and lauric acid). In most of the cases *in vivo* trials focused on feed additives lack of functional screenings of the chosen compounds. Therefore, in my first study the *in vivo* evaluation was preceded by an *in vitro* characterization of the additive. Following *in vitro* digestion, the first results evidenced notable changes in terms of total phenolic content and antioxidant capacity over the 3 differentiated phases. In particular the evidenced data mimicked a sort of peak in gastric release phenolic and antioxidant release followed by a decrease after the intestinal phase. Altogether, the results and the composition of the blend brought the consideration of possible appreciable effects along the entire GIT. After the *in vivo* evaluation, data emphasized that the blended compounds were useful in ameliorating the gut barrier integrity of the jejunum, in which the absorbance of nutrients is much more represented rather than the duodenum. Nevertheless, the MCFAs component, administered via triglycerides forms, was useful to ensure a profuse effectiveness within the gastroenteric tract, as depicted by the morphological development in both duodenum and jejunum. Interestingly, these changes were related also to positive reflexes in terms of salivary cortisol and microbiota composition. Considering the overall data, it seems reasonable to highlight the positive

synergisms among MACFs which acted along the entire enteric tract and phytocompounds (carvacrol and tannic acids) which displayed major reflexes toward jejunum. In addition, the marked reduction of *Clostridium sensu stricto* in gut microbiota evidenced the positive reflexes of the dietary treatment. Nevertheless, differences in composition may manifest limitations when considering additive blend administration. Therefore, it will be important in the future to deeply investigate diversified combinations of additives compounds to gain constant positive reflexes on gut health of weanling piglets.

Pharmacological dosages (2500-3000 mg/kg) of ZnO and dosages of $CuSO_4$ up to 250 mg/kg were commonly administered to counterattack diarrheal disorders during the first two weeks of piglets postweaning phase. The ban on pharmacological trace elements dosages opened the road towards a different perspective on Zn and Cu dietary supplementation. In particular, the necessity to establish a balanced uptake of Zn and Cu bringing appreciable effects on gut health emerged. As evidenced by the literature, the first step was to evaluate the efficacy on more bioavailable sources of both, which highlighted positive feedback on animals gut health throughout numerous studies. Then, acknowledging the dose-dependent characteristics of these formulations reflected the necessity to further optimize Zn and Cu administration. Therefore, the key was to evaluate their biological relationship, which revealed the limits of unbalanced Zn/Cu ratios. In particular, different authors underlined how excesses of Zn over Cu limited Cu availability in different tissues, such as gut, liver and kidneys. The mechanisms behind these interactions are linked to the capacity of excessive Zn to stimulate metallothioneins production in several districts of the organism and, consequently, to enhance their binding activity towards copper ions. Therefore, the hypothesis behind the second study was that an unbalanced administration of Zn and Cu through more bioavailable sources could exert limitations in modulating the gut health of weanling piglets. Thus, the main purpose of the second study of the present thesis was to establish the proper Zn/Cu ratios when considering specialty oxide sources (potentiated zinc and monovalent copper) gaining positive effects on gut health of weaning piglets. The collected data outlined that unbalanced administration of Zn and Cu, in particular doubling Zn over copper (300 mg/kg and 140 mg/kg respectively), can exert limitations in terms of gut health modulation. More specifically, the intestinal barrier permeability and gut local immunity displayed evident alterations both in comparison to a balanced Zn/Cu ratio and pharmacological ZnO (2500 mg/kg). These data were supported by a loss of diversity and richness in fecal microbial populations 14 days after weaning when administering unbalanced Zn/Cu ratios, which represents the end of the most critical period. In addition, taxa evaluation at genus level displayed a higher abundance of potential pathogens (*Escherichia-Shigella*) when considering unbalanced Zn/Cu ratios whereas balanced ratios reduced *Sutterella* and *Clostridium sensu stricto* genera. According to the overall data, results evidenced clear advantages in gut health modulation when supplementing balanced Zn/Cu ratios. The study conducted was useful to gain a step

forward to a better comprehension of Zn and Cu synergisms in conditioning gut health parameters during piglets early weaning phase.

Probiotics have been widely evaluated and applied in both monogastrics and ruminants species to counterattack diarrheal disorders. The well-known effects on intestinal immunity, microbiota and overall integrity have been related to a wide range of lactic acid bacteria, especially during physiological challenges. Nevertheless, the profusion of antimicrobial resistance calls for continuous research on effective strategies. *Weizmannia faecalis* (previously known as *Bacillus coagulans*) has been pointed out as a valid tool to enhance gut health of monogastrics during critical phases. *Weizmannia faecalis* spores are characterized by notable resistance to a wide range of temperature (30-57 °C) and pH (4-10.5) values. For instance, *Bacillus spp*. strains are typically isolated from canned vegetables characterized by low pH values, such as canned tomatoes. Bacillus spp. spores have been reported to markedly condition immunity and antioxidant status of piglets and broilers. One of the main mechanisms of action of these spores relies on the interaction with toll-like receptors within the intestinal lamina propria, which further modulates B-cells activity and, consequently, circulating immunoglobulins level. Similarly, the interaction with TLR might stimulate Nrf2 related pathways and, therefore, condition the antioxidant status of the animal. Nonetheless, information regarding the application of *Bacillus* spp. and *Weizmannia faecalis* in calves nutrition are still inconsistent in the available literature. Therefore, the aim of the third study was to characterize the effectiveness of *Weizmannia faecalis* DSM32016 supplementation on Holstein female calves gut health. *Weizmannia faecalis* spores are more resistant to gastro-enteric environmental conditions than several other lactic acid bacteria strains. Low pH values and variable enzymatic reactions within GIT do not efficiently promote spores leakage. Therefore, a third study was performed with the intention to evaluate the effects of an oral administration of *Weizmannia faecalis* DSM32016 in female Holstein calves. The study was conducted in a local farm during an efficacy trial following EFSA guideline frame to promote the registration of *Weizmannia faecalis* DSM32016 in the European Register of Feed additives. The probiotic was efficient in enhancing calves growth performance while conditioning both the immune and the antioxidant status at the end of the trial. During the critical phase, better fecal scores were highlighted in the treated group. Furthermore, evident effects were detected in terms of beta diversity which outlined a clear differentiation between the two groups. Collected data on microbiota variations and functional metabolic prediction evidenced appreciable differences over time within the two groups. Considering the overall data, it is clear that the effects of *Weizmannia faecalis* administration efficiently drove positive changes in animals gut health, immune and antioxidant status. Nonetheless, it must be considered that *Weizmannia faecalis*, as in the case of many other *Bacillus* strains, is characterized by a rapid lifecycle within the gut environment. This characteristic is desirable when considering probiotic administration in order to avoid possible negative alteration of the gut microbiota. Therefore, it must be outlined that the results obtained were mainly driven by the cumulative presence of *Weizmannia faecalis* spores within the gut lumen. In

conclusion, the present project contributed to optimizing monogastrics and ruminants gut health considering the synergism of diversified compounds, bringing valuable information regarding effective strategies based on blends, trace elements and probiotics. The outlined results brought valuable contributions in the animal nutrition field, especially when it comes to feed supplements optimization.

CHAPTER 11: Acknowledgements

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