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Plant-based strategies to control the zinc and the copper output from swine livestock

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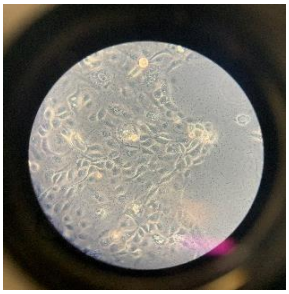
Ph.D. Thesis

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“I am among those who think
that science has great beauty”
Maria Skłodowska-Curie

For my parents

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ABSTRACT

The current state of agriculture, where demand for safe food is increasing rapidly as a consequence of growing population, raises a number of questions related to the one health approach and sustainable animal production with minimal impact on the environment. Swine production is an important branch of food production where weaning is the most vulnerable phase for piglets, often associated with decrease of growth performance and diarrhoea. The maintenance of gut health is therefore a complex endeavour where nutrition is crucial in order to reduce the intestinal disorders. Antimicrobial resistance is also a significant global concern. Reducing antibiotic use in animal production systems decreased prevalence of antibiotic-resistant bacteria in animals about 15%. In the last decade, the European Union banned the antibiotic use as growth promoters in livestock (EU Reg. 1831/2003). The first antibiotic alternative was the wide application of essential nutrients such as zinc (Zn) and copper (Cu) salts in the form of premix in the diets of animals to control digestive disorders. Due to their low bioavailability, Zn and Cu are commonly found in animal' manure as a reflection of their content in the feed. The use of Zn and Cu in feed may also have contributed to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). Despite antibacterial and anti-inflammatory activities, the first adopted alternative against in-feed antibiotics became unsafe due to heavy metal' pollution in livestock wastewater. In order to reduce the high concentration of Zn and Cu and the antibiotic use in animal diets, plant extracts and different phytochemicals are of potential interest due to their antimicrobial, antioxidant and anti-inflammatory properties. However, if nutritional ecology' strategy is not sufficient to reduce the wastewater pollution of heavy metals from livestock production, the development of efficient methods such as multidisciplinary phytoremediation approach is required. First, the preliminary aim was to overview of the role and the main challenges related to the content of essential heavy metals in

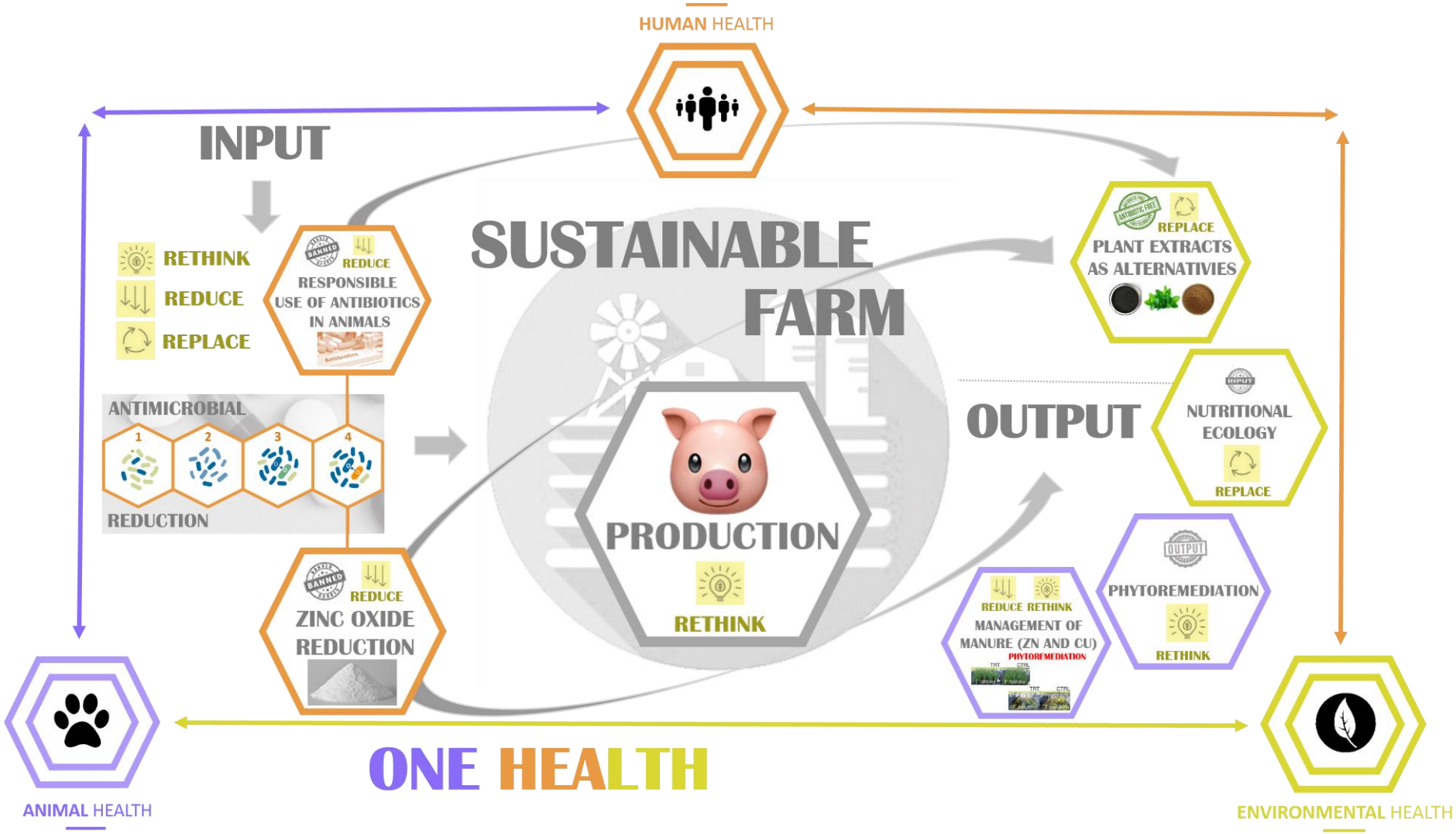
animal feed and to evaluate the concentration of heavy metals from feed and faeces in animal rearing systems in northern Italy. Based on an overview, the main second aim was to develop a plant-based integrated approach to reduce the input and output of both Zn and Cu as well as the use of antibiotic compounds in pig production. Hence, in order to reduce input, the first aim was to test several natural plant-based phytochemicals compounds (tannins and leonardite) *in vivo* and to test of the anti-inflammatory effects of peppermint oil and spearmint oil with porcine alveolar macrophages *in vitro*. The last aim was to assess the ability of two aquatic species, *Typha latifolia* and *Thelypteris palustris* to control the Zn and Cu output from contaminated livestock wastewaters as a cost-efficient phytoremediation strategy. The *in vivo* data revealed that natural plant extracts (leonarditre and tannins) improved animal health. High doses of tannins (1.25%) supplementation showed slight reduction of diet digestibility and protein utilization, however this did not influence on feed intake and growth performance of animals. The inclusion of 0.25% leonardite improved the zootechnical performance, serum lipid profile and gut epithelium integrity, indicating a good general health status. *In vitro* study results showed that both mint oils significantly reduced TNF-α secretion from macrophages. To conclude, leonardite supports an improved stress response in weaned piglets, high dose of tannins did not impair growth performance and both peppermint and spearmint oils had anti-inflammatory activities *in vitro.* Moreover, results obtained from the phytormediation trial showed that *Typha latifolia* and *Thelypteris palustris* can accumulate and translocate Zn and Cu from contaminated wastewater. Thus, phytoremediation was effective to counteract the output of zinc and copper, and possibly other heavy metals from the livestock industry. Hence, an integrated nutritional ecology strategy and phytoremediation approach, in accordance with the modern principles of agroecology is needed to reduce the antibiotics use and heavy metals pollution in food-producing animals. Moreover, plant-based strategy guarantees the improvement of the health status of human and animal and leads to increase of the sustainability in animal rearing systems.

Key words: sustainable agriculture, nutritional ecology, swine production, plant-based phytochemicals and extracts, tannins, leonardite, mint oils, heavy metals, zinc, copper, phytoremediation.

Summary

The current state of agriculture, where demand for safe food is increasing rapidly as a consequence of growing population, raises a number of questions related to the one health approach and sustainable animal production with minimal impact on the environment. Antimicrobial resistance and the wide application of zinc (Zn) and copper (Cu) salts in the form of premix in the diets of animals to control digestive disorders is also a significant global concern. An integrated nutritional ecology strategy based on plant extracts, phytochemicals and phytoremediation approach, in accordance with the modern principles of agroecology is needed to reduce the antibiotic use and heavy metal' wastewater pollution and guarantee the health status of human and animal and leads to increase of the sustainability in animal rearing systems (Figure 1).

Figure 1. Graphical abstract of the thesis.



CHAPTER 1

Scientific background

1.1. The current state in agriculture

The current world population is continuously growing and is expected to increase from 7.3 billion to about 9.7 billion in 2050 (UN, 2017). Demand for safe, nutritious, and healthy food will thus grow rapidly because the population is moving towards more land and water-intensive diets related to the increasing of the consumption of meat and dairy products. The world food production will need to increase 70% by 2050 to meet nutritional needs (Moustafa et al. 2018; FAO 2020). The world's net cultivated area of agricultural crops has yet grown by 11% in the last 50 years and reached 4.8 billion ha (in 2018) mostly at the expense of forest, wetland and grassland habitats. About 0.21 ha of land is cultivated per head of the world's population and the further expansion of cultivated land is limited (FAO, 2020). At the same time, the global irrigated area has doubled due to growing of the cultivated lands, reaching 339 million ha in 2018 (FAO, 2020). Through the global hydrological cycle, about 2 710 km^3 (70%) of renewable water resources is used for agricultural irrigation (FAO, 2011). More effort is needed to increase the use of non-conventional water resources in agriculture, such as the reuse of wastewater, desalinated water, and direct use of agricultural drainage water. However, reuse of nonconventional contaminated wastewater requires the development of novel, efficient, cost-effective, and reliable methods because traditional technologies remain ineffective for providing adequate safe water (Nicholson et al. 2003; Chardon et al. 2012; Alcalde-Sanz and Gawlik, 2017).

Agriculture has an important role in food supply in rural and urban areas and livestock have important socioeconomic functions for rural households, providing a livelihood for more people than any other industry by employing 884 million people (2019). The global value added generated by agriculture, forestry and fishing reached 68% in 2018 (FAO, 2020). Enhancing agricultural productivity contributes to industrial growth by providing cheap labour, capital investment, foreign exchange and markets for manufactured consumer goods (Upton, 2004).

Nevertheless, the global food system and agriculture has an important impact on the environment, through greenhouse gas emissions, water and air pollution including heavy metal' pollution, loss of biodiversity, impacting food security by excessive use of antibiotics and influence on sustainability (Berners-Lee et al. 2018). Crop and livestock activities, together with associated land use, emitted 10.4 billion tonnes of carbon dioxide equivalents of green house gasses into the atmosphere and accounted 52% of the total gas emissions in 2017 (FAO, 2020). Global pesticides used in agriculture reached 4.1 million tonnes in 2018 (FAO, 2020). Moreover, total agricultural use of chemical fertilizers in 2018 reached 188 million tonnes combined by nitrogen (58%), phosphorus (22%) and potassium (21%). The overall fertilizer

use was 40% higher than in 2000 and reached 53 million tonnes in 2018 (FAO, 2020). The current state in agriculture thus raises a number of questions related to the environmental impact of intensified food-producing animals and crops production systems (Moustafa et al. 2018) leading to land and water scarcity (FAO, 2011).

1.2. Concept of one health and sustainable farms in animal production

One health is an interdisciplinary and integrative approach, where human, animal and environmental health are systemically linked (Davis and Sharp, 2020). Moreover, one health approach should design and implement programs, policies, legislation and research to achieve better public health effects (WHO, 2015). Therefore, one health idea should include food safety, the control of zoonoses and combatting antibiotic resistance. In order to meet these challenges, sustainable food production is paramount, and one health is initiated from sustainable animal production. Multidisciplinary teams from academic, government, public and private institutions must work together to achieve these results to support sustainable agricultural practices (Garcia et al. 2020).

One health begins from sustainability which aimed at the best use of environmental services without any negative or harmful impact (Kesavan and Swaminathan, 2008). Sustainable agriculture which is focused on long-term crops and livestock production with minimal impact on the environment, is thus an immediate global priority in order to ensure a balance between food production and the preservation of the environment. In addition, many goals related to sustainable agriculture development and the one health approach need to be effectively implemented in food production. These include (i) water conservation, (ii) a reduction in the use of fertilizers and pesticides, (iii) promotion of biodiversity throughout the entire agro-ecosystem, as well as, (iv) maintain the economic profitability of farms (Pretty 2008; Velten et al. 2015). To make the agricuture and the economy more sustainable with an emphasis on the preservation of natural resources, 17 Sustainable Development Goals (SDGs) were recently adopted. Products, processes and business models therefore need to be redesigned to maximize the value and utility of natural resources, while at the same time reducing adverse health and environmental impacts and climate changes (Hysa et al. 2020).

Nutrition and management are therefore crucial to improve swine rearing and meet sustainable livestock production goals in order to reduce greenhouse gas emissions, water and air pollution including heavy metal' pollution, pesticides and chemical fertilizers use from livestock-related activities (Raubenheimer et al. 2009). As a first step, animals should be fed in accordance with nutritional ecology because livestock nutrition plays a pivotal role in

controlling environmental pollution by improving utilization efficiency of natural resources, and influencing environmental quality by regulating nutrient intake and excretion by animals (Raubenheimer et al. 2009; Hejna et al. 2018).

However, if nutritional ecology strategy is not sufficient to reduce the wastewater pollution from livestock production to ensure the water conservation, the development and local implementation of efficient, cost-effective, reliable and apt materials and methods are required. This can be achieved through multidisciplinary research aimed at studying water pollution for the appropriate management of water resources (Lopez-Alonso et al. 2012; Hejna et al. 2018). Moreover, innovative agriculture practices and technologies are needed to guarantee natural resources availability for future generations (Garcia et al. 2020).

1.3. Antibiotics and antimicrobial resistance in food-producing animals

Antibiotics are natural or synthetic organic molecules that are effective against microorganisms (AlSheikh et al. 2020). Since the first antibiotic, penicillin was discovered (Abraham and Chain, 1940) antibiotic substances are used to treat or prevent animal and human infections by interfering the growth or killing bacteria (WHO, 2015). Antibiotic growth promoters (AGPs) are antibacterial compounds that are added to animal feed or water in subtherapeutic doses for an extended period of time to enhance growth rate and production performance of agricultural animals (Butaye et al. 2003). The enhanced of growth rate may be due to a combination of reduction of normal intestinal flora, which compete with the host for nutrients and decline of harmful gut bacteria, which may reduce performance. The concentration of AGPs used in feed varies with each antimicrobial agent. The subtheraputic dose of AGPs in the gastrointestinal tract of the animal thus inhibit the susceptible bacteria and markedly affect the composition of the bacterial gut flora (Jensen 1998). The benefits of antibiotics as growth promoters were first showed when Stokstad and Jukes (1950) reported that small doses of penicilin could enhance weight gain. AGPs, from several antibiotic classes such as penicillins, macrolides, sulphonamides, tetracyclines, pleuromutilins, polypeptides, streptogramins, carbadox and bambermycin (Milanov et al. 2016) have been used many years thereafter for disease treatment, disease control, disease prevention and increased production efficiency by prevention of bacterial infection in animals, and reduction of the instance of diarrhoea and intestine disorders in animal rearing systems (Van Den Bogaard et al. 2000; Cromwell, 2002).

In light of this, an effort to minimize economic losses, antibiotics effective against bacteria are the most commonly prescribed drugs in the animal industry (Landers et al. 2012).

In farms, the antibiotics are applied to the animals in the form of in-feed supplements due to the higher efficiency compared with administering individual treatments. Therefore, wide administration of AGPs is a major contributor to development and occurrence of antimicrobial resistance (AMR) in animals and humans (Rousham et al. 2018).

Bacteria have genetic plasticity that allows them to respond to different environmental threats such as the presence of antibiotics. Bacterial cells derived from a susceptible population develop genetic mutations that negatively affect drug activity causing cell survival in the presence of antimicrobial molecules (Munita et al. 2016). Resistance to antimicrobials help microbes in their survival against other antibiotic-producing microorganisms in the surrounding environment (Phillips et al. 2004). Thus, some infections are caused by microorganisms that fail to respond to antibiotics treatments because microorganisms develop resistance to the antimicrobial against which they were originally vulnerable (Jindal et al. 2015).

Antibiotics applied in food-producing animals are related to those used in human medicine and can select for resistance in these animals. Cross species transmission of resistant bacteria or resistant genetic elements from animals to humans can occur through contaminated animal retail products and can be surpassed between animals and farmworkers influencing at human health (Dewey et al. 1997; Landers et al. 2012; Tang et al. 2017). Moreover, infections with antibiotic resistant bacteria result in increased mortality, morbidity, and economic costs (Cosgrove and Carmeli 2003). Therefore, WHO (2020) has declared that AMR is a risk for both human and animal health and AGP' usage has been banned in many countries starting in Sweden (1986), Denmark (1998), European Union (EU, 1831/2003) and recently the USA decided to withdraw the usage of AGPs (2017).

Moreover, antimicrobial resistance will attribute to a 10 million deaths per year globally by 2050 (O'Neill et al. 2015). Thus, Tang et al. (2017) demonstrated that reducing antibiotic use in animal production systems decreased prevalence of antibiotic-resistant bacteria in animals about 15% and multidrug-resistant bacteria about 24-32%. Moreover, a study conducted by Aarestrup et al. (2010) showed that the improvement in productivity was observed, even if antibiotics consumption per kilogram of pig produced in Denmark decreased by around 50% from 1992 to 2008 (Figure 2). Long-term swine productivity was thus not negatively impacted by a ban on AGP use and the change in antibiotics consumption (Aarestrup et al. 2010).

Therefore, the state of livestock has changed significantly, where the industry has focused on sustainable meat production, which represents a shift from antibiotics usage (Zeineldin et al. 2019). New nutrition strategies to account for decreased antibiotic use should be related with

(i) high quality nutrition and a well-managed environment, (ii) high quality, cost-efficient and palatable of dietary ingredients, (iii) intestinal microbial ecosystem balance (eubiosis), (iv) functional additives and ingredients and (v) innovations.

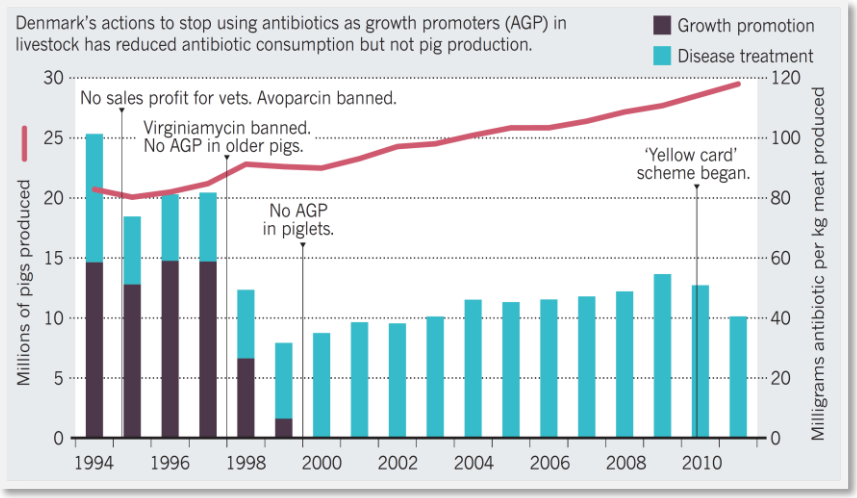


Figure 2. Consumption of antimicrobials for use as AGPs (viola bars) or for therapeutic administration (blue bars) from 1992 to 2010 by the Danish swine production system. Outbreaks of PRRS (1996 to 2000), disease attributable to *Lawsonia intracellularis* (1998 to 2002), and PMWS (2001 to 2006) are indicated (arrows). *Adapted from Aarestrup (2012).

1.4. Swine production

Meat is an important source of nutrition and its global production tripled over the past 50 years reaching 342 million tonnes in 2018. Moreover, according to FAO (2020) prediction, meat production' demand will be continuously growing in the next years (Table 1). Many species are bred for meat but only pigs, poultry and cattle accounted for nearly 90% of global meat production from 2000 to 2018 (FAO, 2020). Pig meat is the most produced type of meat representing 35% of total meat production in 2018 with more than 120 million tonnes of produced pork (FAO, 2020). Moreover, swine production provides to market not only fresh meat but also secondary products. The rapid growth of the swine production sector contributed to high economic gain due to the relatively short life cycle of pigs that have a high feed conversion ratio and reproductive rate. Half of the commercial pig rearing system is highly intensive where pigs are bred indoors and are distributed into several breeding phases including gilts or sows and animals reared for meat, such as piglets, weaning, growing and finishing pigs.

In intensive livestock production, weaning is one of the most challenging and critical phase which exposes piglets to a combination of biological stressors related to physiological, environmental and social challenges. In particular, stressors include, (i) an abrupt separation from the sow, (ii) transportation and handling stress, (iii) a different food source, (iv) social hierarchy stress, (v) co-mingling with pigs from other litters, (vi) a different physical

environment with increased exposure to pathogens and dietary or environmental antigens (Rossi et al. 2013; Rossi et al. 2014a). Weaning thus is often associated with low feed intake which impairs growth performance, causes fluctuations and dysfunction in gut function and predisposes piglets to digestive disorders, nutrient malabsorption and consequently diarrhoea, which is often associated with *Escherichia coli* infection of the intestine (Kim et al. 2019). The main consequence of those stressors is increased mortality rate of piglets (Moeser et al. 2017).

Table 1. Expected changes in the world's meat production (in a million tons of carcass weight) over the next years.

	2018	**2020**	**2021**	**2022**	**2023**	**2024**	**2025**
Beef and veal							
Production	67 354	71 140	71 698	71 910	72 429	72 929	73 525
Pig meat							
Production	120 881	106 526	109 006	112 829	116 274	119 869	123 466
Poultry meat							
Production	114 267	132 067	133 301	134 561	136 116	137 666	139 256

* Data from 218 year adapted from FAO (2020) and from 2020–2025 years from OECD-FAO (2020) "OECD-FAO Agricultural Outlook".

The advanced breeding technologies thus have to be improved in order to maximize the production and reduce its cost. Hence, management, genetics, animal nutrition and feeding combined with protection of the animal welfare, housing and environmental requirements, and reproductive efficiency need to be largely applied in order to improve swine rearing and meet the sustainable livestock production goals and modern multidisciplinary principles of agroecology (Raubenheimer et al. 2009).

1.5. Gut health of animals

Gut health can be described as a condition of homeostasis in the gastrointestinal tract, with respect to its overall structure and function (Pluske et al. 2018). The gastrointestinal tract is therefore a complex and dynamic organ which plays a pivotal role as a first physical barrier against external factors and pathogens (Chelakkot et al. 2018; Figure 3). The internal lining of the gut is composted by one single layer of intestinal epithelial cells. The intestinal epithelial monolayer is composed of different cells such as enterocytes, for which the major function is the maintenance of epithelial barrier integrity which allows the permeability of essential ions, nutrients, and water but restricts the entry of bacterial toxins and pathogens (Rescigno et al. 2011; Kagnoff et al. 2014). Intestinal epithelial cells are also capable of phagocytosing bacteria and can regulate the inflammatory response against bacterial toxins (Kagnoff et al. 2014).

Moreover, the epithelial cell layer together with the chemical barrier of the mucosal layer and the cellular immune system maintain a symbiotic relationship with commensal bacteria. Different commensal bacteria reside in the gastrointestinal tract have a vital role in digestion and the development of the immune system, but they also present the risk of infection. Gut microbiota, consisting of many bacteria and viruses, are pivotal for the maintenance of a symbiotic relationship with immune cells (Kahrstrom et al. 2016). The population and activity of bacteria in the gut is influenced by several factors such as the structure and composition, solubility and the amount and type of substrate available affects the gut microbial ecology (Hogberg and Lindberg, 2004; Konstantinov et al. 2004; Bindelle et al. 2010).

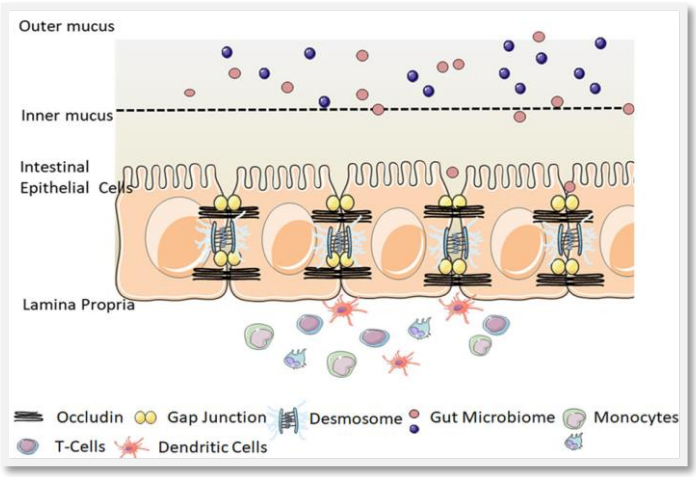


Figure 3. Intestinal epithelial barrier. Epithelial cells form a layer that functions as a physical barrier facilitated by tight connections between each cell. A number of tight junction protein components seal the paracellular pathway and conduct gate and fence functions. The mucosal layer is a chemical barrier that is critical to limit the contact between the microbiome and epithelial cells. *Adapted from Chelakkot et al. (2018).

The maintenance of epithelial barrier integrity is strictly connected with the tight junction proteins (Cereijido et al. 2007). The intracellular signalling transduction system dynamically modulates the tight junction protein complexes, allow the transport of essential molecules and block harmful substances. An imbalance in these regulations leads into an uncontrollable immune reaction in the intestinal environment or allows the uncontrolled growth of microbiota, which leads to compromised barrier integrity and is linked with pathological conditions, different inflammatory diseases and metabolic disorders (Chelakkot et al. 2018).

Furthermore, the functions of the gastrointestinal tract extend beyond the processes such as feed intake, digestion, and the subsequent active or passive absorption and barrier function. The association between the enteric nervous system and the higher ceneres via the parasympathetic nervous system and endocrine system also plays a key role in animal well-being, health, and structure and function of the gastrointestinal tract (Pluske et al. 2018).

In neonatal pigs, the mucosal epithelium of the small intestine is immature, however still protected by maternal immunity. The critical window of postnatal gastrointestinal development intensifies at weaning, (i) when a colonization of the gut occurs by new microorganisms entering the alimentary canal with solid feed and (ii) when rapid changes in size, protein turnover rates, microbiota mass and composition, and quick alterations in digestive absorptive, barrier and immune functions occurs in gastrointestinal tract (Domeneghini et al. 2006; Moeser et al. 2017; Figure 4). This frequently exposes piglets to diarrhoea occurrence and other intestinal disturbances (Domeneghini et al. 2006; Adewole et al. 2016). The period immediately post-weaning is thus the most vulnerable period in the pig's life, contributing to an intestinal inflammatory status that in turn compromises villous crypt architecture, gastrointestinal barrier function and disruption of the microbiota (Pie et al. 2004; Gresse et al. 2017; Moeser et al. 2017). Maintaining animal performance and health by modulating the microbial ecology in the digestive tract is thus the most crucial part of animal production systems (Pluske et al. 2018).

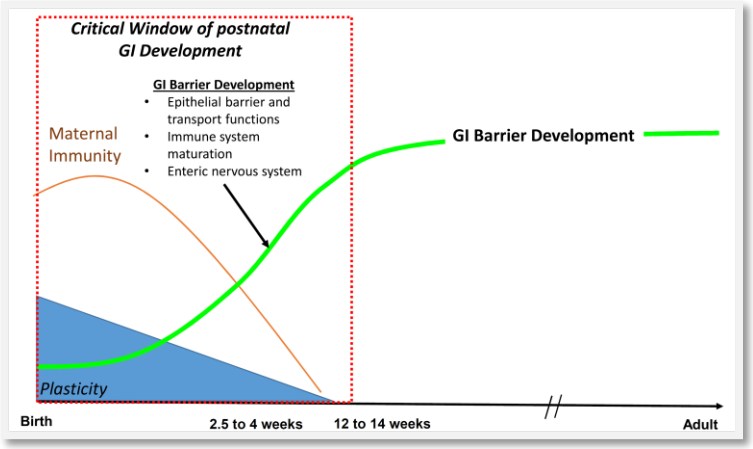


Figure 4. Ontogeny of postnatal gastrointestinal (GI) barrier function development in the pig. During the first 12 weeks of postnatal life, the GI system in the pigs undergoes significant development. Colostrum and sow's milk initially provides the piglet protective passive immunity as well as important growth and immune factors. The postnatal period is marked by maturation of the epithelial barrier and transport functions, and immune and enteric nervous systems (indicated by green line) that are almost complete by 12 to 14 weeks of age. *Adapted from Moeser et al. (2017).

The maintenance of gut health is thus a complex phenomenon and relays at a delicate interaction between components including, (i) a stable and appropriate function of gut microbiota and microbiome, (ii) a stable and appropriate microbial population, (iii) functional and protective gut barrier, (iv) defense mechanism of mucosal immune, (v) the absence of disease, (vi) minimal activation/stimulation of stress/neural pathways and (vii) digestion and absorption of nutrients and energy generation (Montagne et al. 2003; Pluske et al. 2018).

1.6. First scenario: alternatives to control antibiotics input in swine nutrition

Livestock antibiotic resistance led the European Union to ban the use of antibiotics as growth-promoting agents (EU, 1831/2003). The European Food Safety Authority (EFSA, 2017) also recommended specific measures focused at reduce, rethink and replace of the antibiotic use in animals. Particularly, (i) reducing antibiotics use in animal production to the minimum necessary by setting targets for reducing the use of critically important antibiotics, increasing responsibility of veterinarians and using antibiotics only when needed, (ii) rethinking the livestock production system by prevention and control of diseases in animals, considering alternative farming systems and offering education to people related to animal industry, (iii) replacing them with alternative treatments by considering new antibiotics alternatives.

1.6.1. Different alternatives to antibiotics in food-producing animals

Alternatives to antibiotics are thus substances that can be administered as therapeutic drugs and ideal antibiotic alternatives should have the similar beneficial effects of antibiotics (Seal et al. 2013). Ideal alternatives to antibiotics should thus (i) not induce bacterial resistance, (ii) kill or inhibit the growth of pathogenic bacteria, (iii) not affect palatability, (iv) be stable in the feed and animal gastrointestinal tract, (v) have no toxic or no side effects on animals, (vi) not destroy the normal intestinal flora of animals, (vii) enhance the body resistance to the disease, (viii) improve feed efficiency and promote animal growth, (ix) have good compatibility, (x) be easy to eliminate from the body or consist of short term of residues and (xi) be easily decomposed and not affect the environment. In fact, currently there are no alternatives to antibiotic that meet all mentioned requirements (Cheng et al. 2014).

Due to EU restrictions and EFSA recommendation, alternatives to antibiotics feed ingredients and additives were urgently needed to guarantee animal production in accordance with health principles. A number of alternatives have been thus proposed to overcome the increase in the mortality and morbidity of farm-producing animals (Seal et al. 2013). These include (i) bacteriophages and their lysins, (ii) antimicrobial peptides, (iii) probiotics and prebiotics, (iv) inhibitors, (v) feed enzymes, (vi) plant extracts and (vii) antibacterial immunostimulants with bioactive functions such as vaccines, vitamins, oligosaccharides, polysaccharides, amino acids, hormones, bacterial extracts and mineral substances, that improve the immune function and consequently the host's resistance to diseases (Millet and Maertens, 2011; Cheng et al. 2014).

Among different antibiotics alternatives (i) bacteriophages are viruses that are parasitic on bacteria, and they are able to treat bacterial infections by replicate in host cells and produce new lytic phages (Wittebole et al. 2014); (ii) antimicrobial peptides are nonribosomally

synthesized AMPs and ribosomally synthesized AMPs which are able to destroy the bacterial cell membranes thus are effective against many Gram-negative bacteria and Gram-positive bacteria; (iii) probiotics are able to destroy pathogenic microorganisms by producing antimicrobial compounds to improve gastrointestinal microbial environment and prebiotics are non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract and selectively proliferate intestinal bacteria and show antiviral activity (Gibson et al. 2004; Cheng et al. 2014); (iv) inhibitors can block the functions of QS system which regulate bacterial pathogenicity and therefore prevent bacterial virulence regulated by this system (Swift et al. 2001); (v) enzymes influence the absorption of nutrients but also produce nutrients for specific populations of bacteria through their action therefore, their use has a direct impact on the microfloral populations (Apajalahti et al. 2004 Bedford and Cowieson, 2012); (vi) immunostimulants which directly initiate activation of innate defense mechanisms acting on receptors and triggering intracellular genes that may result in the production of antimicrobial molecules (Cheng et al. 2014), thus immunostimulants as feed additives such as mineral substances can improve the innate defense of animals (Bricknell and Dalmo, 2005). Therefore, the first widely adopted alternative against in feed antibiotics was the application of high doses of mineral substances, which are also heavy metals such as zinc (Zn) and copper (Cu) salts in the form of premix due to their antibacterial and anti-inflammatory activities.

1.6.2. Heavy metals as alternative to antibiotic use in animal nutrition

Heavy metals (HMs) are metallic elements that have a high density compared to water and induce toxicity at low exposure levels (Bhargava et al. 2012; Govind and Madhuri, 2014; Dai et al. 2016; Giromini et al. 2016; Santos et al. 2018). Different HMs can enter animal diets both as contaminants or undesirable substances and as essential nutrients (Fink-Gremmels, 2012; Hejna et al. 2018 Table 2). Some heavy metals are essential to maintain biochemical and physiological functions, although excessive exposure with higher concentrations of these elements has been linked with cellular or systemic disorders, acute and chronic toxicity, and sources of pollution (Rossi et al. 2014b). In the farming industry, essential trace elements are therefore usually used as feed additives in order to not only satisfy the nutritional requirements and prevent nutritional deficiencies, but also to promote health and welfare, optimize production and improve food safety (Suttle, 2010). These elements should thus be included within the animal diets as mineral additives in compliance with the maximum admitted levels (EC˚N 1831/2003; Lopez-Alonso et al. 2012).

Heavy metals can also enter animal' diets as contaminants with no established biological functions (Reg. 2002/32/EC). Arsenic (As), cadmium (Cd), chrome (Cr), lead (Pb) and mercury (Hg) are a major risk to public health in high toxicity and can induce organ damage even at low exposure levels (Table 2). In line with previous research (Hejna et al. 2019), swine and cattle diets were not affected by high amounts of the undesirable elements.

Table 2. Heavy metals in animal nutrition (Govind and Madhuri, 2014; Hejna et al. 2018).

Essential elements (authorized in animal nutrition according to EC N°1831/2003)								
Co (cobalt)	Cr (chromium)	Cu (copper)	Fe (iron)	Mn (manganese)	Mo (molybdenum)	Ni (nickel)	Se (selenium)	Zn (zinc)
Nonessential elements (undesirable elements according to 2002/32/EC)								
	As (arsenic)			Cd (cadmium)		Hg (mercury)		Pb (lead)

1.6.3. The importance of zinc and copper use in animal feeding

Zinc (Zn) is one of heavy metal toxic to animals, bacteria and plants when encountered in high concentrations however it is also essential in the maintenance and restoration of barrier integrity, protection against pathogens, modulation of the immune system and promoting antibody production against intestinal pathogens. The role of zinc can be grouped into catalytic, structural and regulatory functions (Cousins et al. 1996; Rossi et al. 2014a). Zinc is required for the structural and functional integrity and almost every signalling and metabolic pathway is dependent on zinc proteins (Cousins et al. 2003). The most important functions of Zn in livestock are related to (i) gene expression, where zinc regulates genes involved in signal transduction, responses to stress, growth and energy utilization, (ii) appetite control where zinc increases the expression of the gene for the appetite-regulating hormone cholecystokinin (Cousins et al. 2003), (iii) fat absorption and (iv) antioxidant defence where zinc deprivation increases the susceptibility of endothelial cells to oxidant stress (Beattie and Kwun, 2004; Suttle 2010). Today, zinc oxide (ZnO) which is the most common form of Zn is widely used to maintain the nutritional requirements of weaning (to 150 ppm in complete feed, EU 1334/2003). In addition, Zn is applied at pharmacological doses (from 1000 to 3000 mg/kg feed) as an alternative to antibiotic to promote growth performance and to control enteric intestinal bacterial disorders as well as enhancing the immune system for diarrhoea prevention in pigs (Sales, 2013; Walk et al. 2015).

Copper (Cu) is also an important essential mineral for the activity of numerous enzymes cofactors and reactive proteins. Copper proteins are responsible for (i) cellular respiration by terminal electron transfer in the respiratory chain and thus for energy generation in tissues, (ii)

protection against oxidant stress from free radicals, including those generated during respiration and (iii) iron transport (Suttle 2010). Copper is widely supplemented in the diet of weaning pigs due to its role in increasing growth performance and favouring a better feed conversion ratio (Polen and Voia, 2015). In pigs, dietary concentrations from 150 to 250 mg of Cu/kg can maximize growth performance without exposing animals to any risk of poisoning (Suleiman et al. 2015). The routine inclusion of $CuSO_4$, which is the most common form of Cu in diets was found to reduce intestinal diseases and to be a cost-effective solution to the replacement growth-promoting antibiotics in pig diets.

1.6.4. Heavy metals and their impact on environment

Considering that the bioavailability and digestibility of essential mineral sources are limited, the metals are only partially digested by animals and the excess is eliminated by excretion in faeces and found in the manure (Adewole et. al. 2016). The HM content in manure is therefore its reflection of the feed (Nicholson et al. 2003; Chardon et al. 2012). The study by Hejna et al. (2019) indicated that Zn and Cu are widely found in pig manure as results of their high doses in swine diets. Through the manure, large amounts of metallic ions may enter livestock wastewater (Gul et al. 2015). The HM contamination of wastewater (Nicholson et al. 2003; Moral et al. 2005) leads to severe agricultural soil contamination (Figure 5). This caused (i) the deterioration of agricultural land, (ii) the decreasing quality of agricultural soils, (iii) the reduced the quality of cultivation, (iv) phytotoxicity, (v) disequilibrium in the soil microbial processes, (vi) eutrophication and (vii) drastically reduces potential use of wastewater for agricultural irrigation (Nicholson et al. 2003; Moral et al. 2005; Luo et al. 2009; Lopez-Alonso et al. 2012; Zhang et al. 2012; Jakubus et al. 2013, Gul et al. 2015).

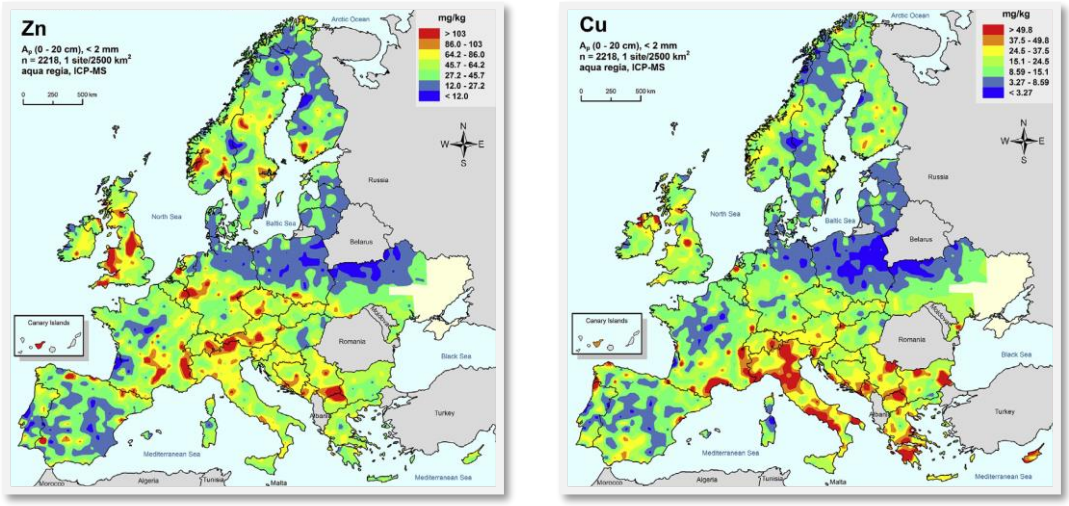


Figure 5. Regional distribution of the Zn and Cu in the GEMAS agricultural soil samples from Europe. *Adapted from Reimann et al. 2014 and Reimann et al. 2018.

The anthropogenic contamination of the environment with HMs is therefore a serious problem and their long-term accumulation in the environment has led to their propagation in the food chain by accidental soil ingestion, contamination of edible plants through the soil or the consumption of contaminated animal derived food products (Hejna et al. 2018; Figure 6).

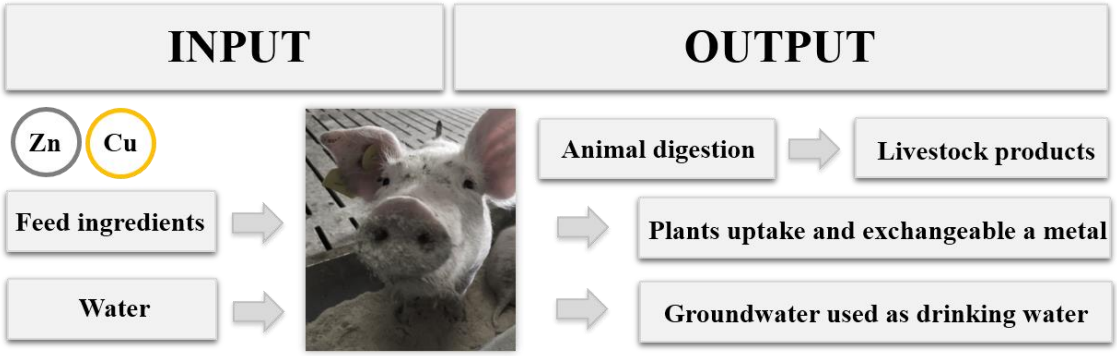


Figure 6. Possible routes of HM entrance to the food chain and the consequences of their output.

The focus of EU environmental protection policies is on promoting economic growth together with reducing the impact of heavy metals. The comprehensive regulations on the maximum authorized admissible concentrations of essential and undesirable substances of essential trace elements in additives have been established for animal nutrition (EC Reg. No 1831/2003; Dir. 2002/32/EC). The EU also recently banned the inclusion of pharmacological levels of zinc oxide in animal feed after 2022 (EMA/394961/2017) due to overall benefit and risk balance remains negative for feed additives containing zinc oxide. Similarly, the new maximum admissible level of Cu content (for different Cu sources) was also established in complete feed for different animal species (EU Regulation, 2018/1039) in order to protect feed and food safety and ultimately human health. In light of this, nutrition plays a crucial role in the ecology of minerals' and the sustainable development of swine production.

1.7. New strategy: plant-based alternatives to antibiotics and Zn and Cu input in swine livestock

The first adopted alternative against in-feed antibiotics was the wide application of high doses of zinc and copper salts in the form of premix which, despite their antibacterial and anti-inflammatory activities, raised many concerns related to environmental pollution. The use of zinc and copper in feed may also have contributed to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) due to the potential increase in the prevalence of antibiotic resistant bacteria and concern that MRSA may become a zoonotic pathogen in animal production (Lyubenova et al. 2011; Rossi et al. 2014a). Hence, innovative compounds,

alternatives to antibiotics and to ZnO and different sources of Cu are required to prevent enteric disorders during weaning and to enhance the gut health. Many alternatives with antimicrobial, anti-inflammatory and antioxidant properties are studied to promote gut health of animals. One of the alternative may be natural plant-based extracts and phytochemicals such as tannins, leonardite and mint oils (Onelli et al. 2017).

Plant extracts are secondary plant metabolites and can be obtained naturally from parts of plant materials, such as flowers, buds, seeds, leaves, twigs, bark, wood, fruits, and roots. Phytochemicals are natural bioactive compounds that occur naturally in plants and are incorporated into animal feed to enhance productivity (Huang et al. 2016; Gadde et al. 2017). Different plant extracts and phytochemicals demonstrate a wide spectrum of antibacterial activities with different modes of action such as (i) contain phenolic compounds, which owns strong antibacterial properties (Lambert et al. 2001), (ii) directly kill bacteria due to their hydrophobicity (Carson et al. 2002), (iii) active components disturb the enzyme system of bacteria and block their virulence (Ankri and Mirelman, 1999) and (iv) bioactive components may prevent the development of virulence structures in bacteria (Burt et al. 2007) thus make them effectiveness against microbes (AlSheikh et al. 2020; Figure 7).

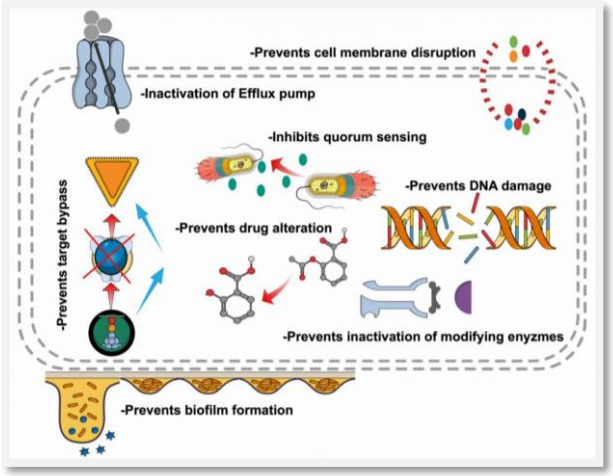


Figure 7. Phytochemicals as antimicrobials: mode of action and their effectiveness against microbes. *Adapted from: AlSheikh et al. (2020).

Moreover, plant extracts and phytochemicals are of potential interest due to their beneficial antiviral, antioxidant and anti-inflammatory properties (Baydar et al. 2004; Dundar et al. 2008; Liu et al. 2012). Different *in vitro* and *in vivo* studies have demonstrated that plant extracts and phytochemicals can maintain animals in good health through direct suppression of the proliferation of pathogens, alteration of gut microbial populations and enhancement of immune functions (Lee et al. 2004). In addition, their inclusion in the diets alters and stabilizes the intestinal microbiota and reduces microbial toxic metabolites in the gut, which improve

performance (Kim et al. 2010). All together, may lead to the ability to use plant extracts and phytochemicals as in-feed antibiotic alternatives in diets to improve performance, health and welfare of farm animals (Stein and Kil, 2006).

1.7.1. Tannins as bioactive phytoextract to counteract with wide use of antibiotics and Zn

The main bioactive compounds of the phytochemicals are polyphenols and tannins are water-soluble polyphenolic secondary metabolites (Khanbabaee and van Ree, 2001) derived from different plant sites such as wood, leaves, and seeds and supplemented to animals as additives (Huang et al. 2018). Tannins have been tested in livestock initially as tannin-rich feedstuffs such as sorghum, barley, maize and fava beans, and more recently as tannin extracts from different plants such as grape seed, grape pomace, acorns, oak, green tea leaves, and pomegranate (Cappai et al. 2012; Hao et al. 2015). The commercial products are mostly derived from chestnut (*Castanea sativa Mill*.) and quebracho (*Schinopsis Balansae*) plants and are listed as authorized additives in feeding stuffs (EC regulation 1831/2003; 2004/C50/01).

The antimicrobial, antioxidant and anti-inflammatory activities of tannins supplementation could alleviate post-weaning diarrhoea and enhance growth performances (Steendam et al. 2004; Biaga et al. 2010; Girard et al. 2019). Beneficial effects of tannins supplementation in piglets were also found at intestinal level (Brus et al. 2013b; Girard et al. 2019). Tannins have been tested in intensive swine farms (Steendam et al. 2004) and different literature cases have shown contradictory and heterogeneous results (Biaga et al. 2010; Girard et al. 2019) related to the dosages of tannin inclusion. The inclusion of 1%, 2% and 3% of chestnut and quebracho or chestnut tannins had no effect on average daily gain (ADG), body weight (BW) and feed efficiency in pigs (Candek-Potokar et al. 2015; Girard et al. 20018). Bee et al. (2017) reported that the inclusion of 3% Ch/Qu tannins significantly decreased the gain-to-feed ratio in boars, while BW and ADG were not influenced. Moreover, lower doses (from 0.11% to 0.45%) of chestnut tannins did not improve growth performance in piglets (Brus et al. 2013a). Biaga et al. (2010) presented that the inclusion of 0.45% of tannin showed higher average daily gain and increased feed efficiency in pigs. Tannins supplementation at 1% and 2% of inclusion in the diet showed a positive effect on daily feed intake and average daily gain of pigs (Girard et al. 2018) and the concentrations from 0.11% to 0.45% of chestnut tannins in swine diets improved feed efficiency (Parys et al. 2010). These contradictory results could be related with tannins' ability to bind proteins and carbohydrates causing feed palatability and digestibility decrease and depression of digestive capacity in the small intestine (Huang et al. 2018). Moreover, the combined effect of chestnut and quebracho (Ch/Qu) tannins could be exacerbated during stressful conditions, such as experimental bacterial infections (Girard et al.

2018; Girard et al. 2019). According to Reggi et al. (2020), beneficial effects were reported when Ch/Qu digesta were administered to experimentally stressed intestinal swine cells. Thus, the effective supplementation of tannins on pigs' performance is related to the source of tannins (Ch and Qu), dosage of tannins, and the type of tannins included in the diets.

1.7.2. Leonardite as bioactive phytoextract to counteract with wide use of antibiotic and Zn

Leonardite is a microbial-derived product mainly composed of humic acids (HAs), which are derived from the decomposition of organic matter, usually exploited for the fertilization of soil. HAs may provide benefits to piglets' health during post-weaning, protect the mucosa of the intestine, with recognized anti-inflammatory, antiphlogistic, adsorptive and antitoxic proprieties (Aksu and Bozkurt, 2009; Ozturk et al. 2012; Islam et al. 2013; Trckova et al. 2017; Domínguez-Negrete et al. 2019). HAs have shown antioxidant proprieties that could sustain the animals during the stressful period of weaning, antimicrobial activity against pathogens leading to a decreased incidence of diarrhoea and better growth performance also modulating the animal's metabolism (Wang et al. 2008; Aeschbacher et al. 2012). Humic acids and their sodium salts are permitted for oral use (from 500 to 2000 mg/kg of body weight) in horses, ruminants, swine and poultry for the treatment of diarrhoea, dyspepsia and acute intoxications (EGTOP/1/2011). Lower levels of humic substances used as a feed additive (from 2 to 10 g/100g of diet) in the pigs' diet improve growth performance and meat quality, also reducing ammonia emissions from manure (Ji et al. 2006; Wang et al. 2008; Kim et al. 2019).

1.7.3. Mint oils as alternative to antibiotic use and their anti-inflammatory effect

Peppermint (*Mentha piperita L.)* and spearmint (*Mentha spicata L.*) belong to the mint genus and are known for high content of essential oils deposited in the glandular trichomes (Dhifi et al. 2016; Kalemba and Synowiec 2020) and abundant content of phenolic compounds (Park et al. 2019; Wu et al. 2019). Mint oils as feed additives are therefore being investigated for promoting health in piglets due to their anti-inflammatory properties (Omonijo et al. 2018). Moreover, the effects of mint oils on the immune response of pigs reared under disease challenge conditions have not been fully revealed. Porcine alveolar macrophages (PAMs) are lung tissue-resident professional phagocytes in pigs and play important roles in immune response (Li et al. 2017; Wang et al. 2017; Bordet et al. 2018). Lipopolysaccharide (LPS) challenge could induce pro-inflammatory cytokines secretion from PAMs (Arango Duque et al. 2014; Wang et al. 2017), therefore, culturing PAMs with LPS has been widely used to test the *in vitro* anti-inflammatory effects (Islam et al. 2013; Murphy and Weaver, 2017).

1.8. New strategy: reducing the output of heavy metals from swine livestock wastewater through plant-based approach

Animals should be fed in accordance with nutritional ecology strategy because livestock nutrition plays a pivotal role in controlling environmental pollution (Raubenheimer et al. 2009, Hejna et al. 2018). However, if the nutritional ecology strategy is not sufficient to reduce the wastewater pollution from livestock production, to ensure water conservation, then efficient, cost-effective, reliable, and apt materials and methods need to be developed and locally implemented (Lopez-Alonso et al. 2012).

In swine farms, heavy metal contamination of wastewater considerably reduces its potential for being recycled in irrigation (Moral et al. 2005; Hejna et al. 2018). The various physical and chemical traditional wastewater treatment technologies are available, but most of them are costly, labour-intensive and cause lower fertility of soil and thus, are ineffective in providing adequate, safe water. Therefore, in order to increase the sustainability in animal production there is a need for a simple solution for proper management of water resources. One promising possibility is to bioaccumulate of metals from the soil and wastewater through the process of phytoremediation. According to Bhargava et al. (2012) phytoremediation is simple clean-up technology which removes, degradates and immobilizes metals from different matrices such as sludges, soil, sediments, groundwater and wastewater through the use of plants that accumulate large amounts of heavy metal contaminants. Depending on the conditions, plants used and the contaminants, phytoremediation can be divided into (i) phytoextraction, (ii) phytofiltration and (iii) phytostabilization (Thangavel and Subbhuraam, 2004; Ali et al. 2013). Phytoextraction is exploited by plants to uptake metals inside the roots or underground organs, and to translocate and to accumulate them in aboveground tissues (Małachowska-Jutsz and Gnida 2015; Yan et al. 2020). Phytofiltration includes the use of plant roots (rhizofiltration), shoots (caulofiltration) and seedlings (blastofiltration). Phytostabilization uses plants to immobilize metals in the substrate or in the rhizosphere and preventing their leaching to groundwater (Seth 2012; Ali et al. 2020; Yan et al. 2020). Phytoremediation has gained acceptance in the last years as simple, acceptable, cost-effective, green, efficient, noninvasive and novel complementary technology for engineering-based remediation methods (Bhargava et al. 2012; Ali et al. 2013).

1.8.1. Phytoremediation through plant species

Plants adopt both avoidance and tolerance mechanisms to deal with the toxicity of heavy metals (Małachowska-Jutsz and Gnida 2015; Yan et al. 2020). Avoidance is the first line of defense, and plants limit the uptake of heavy metals and their entry in the root tissues (Dalvi

and Bhalerao 2013). The mechanisms of avoidance work at different levels and involve (i) cell wall modification through callose, suberin or lignin deposition (Miransari 2011), (ii) the sequestration of metals into the cell wall (Memon and Schröder P. 2009; Krzeslowska, 2011), (iii) the secretion of a root extracellular matrix which binds ions, stabilizing heavy metals in the rhizosphere and limiting their assimilation (Dalvi and Bhalerao 2013) and (iv) the removal of excess metals by leaf glands (Małachowska-Jutsz and Gnida 2015). Tolerance mechanisms enable plant cells to accumulate metal ions in cell walls and vacuoles after chelation by amino acids, phytochelatins, metallothioneins, pectins and phenols (Yadav 2010; Hasan et al. 2017).

The most useful method for phytoremediation of livestock manure and wastewaters can be achieved through constructed wetlands of aquatic plants, which uptake metals and organic matter from water and mimic natural wetland processes at biological, chemical and physiological levels (Stefanakis et al. 2014; Kadlec et al. 2000). Constructed wetlands can recover contaminants mainly due to the removal capability of microorganisms and to the pollutants' adsorption from substrate. Plants are able to extract contaminants through the root system and improve pollutant removal by providing an appropriate environment for rhizosphere microorganism growth (Ali et al. 2020; Kadlec et al. 2000).

Elements such as Cu, Mo, Ni and Zn are necessary for plant growth in low concentrations. However, beyond certain threshold concentrations, these elements become toxic for most of the plant species. The discovery that certain plant species (hyperaccumulators) are capable to uptake high concentration of heavy metals from soil or water, due to the roots' ability to adsorb and translocate these compounds in plant cells, opened new possibilities to use plants to remediate contaminated matrices (Peralta-Videaa et al. 2002). Some hyperaccumulator species, such as aquatic *Typha latifolia* (Broadleaf cattail) and *Thelypteris palustris* (Marsh fern), are thus widely used for phytoremediation processes of contaminated water (Lee et al. 2004; Meers et al. 2005; Manousaki and Kalogerakis 2009; Chandra and Yadav 2010; Klink et al. 2013; Hazra et al. 2015; Almeida et al. 2016; Salem et al. 2017; Hejna et al. 2020; Knight et al. 2000; Stroppa et al. 2020). The proper hyperaccumulator plants for removal of heavy metals should thus have the following components: (i) high growth rate, (ii) highly branched and widely distributed root system, (iii) good adaptation to prevailing environmental and climatic conditions, (iv) easy cultivation and harvest, (v) production of more above-ground biomass, (vi) resistance to pathogens and pests, (vii) more accumulation of the target heavy metals from soil, (viii) translocation of the accumulated heavy metals from roots to shoots and (ix) tolerance to the toxic effects of the target heavy metals (Sakakibara et al. 2011; Shabani and Sayadi 2012; Ali et al. 2013; Maric et al. 2013).

1.9. References

Aarestrup FM, Jensen VF, Emborg HD, Jacobsen E, Wegener HC. 2010. Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark. American Journal of Veterinary Research. 71(7):726–733.

Aarestrup. 2012. Sustainable farming: get pigs off antibiotics. Nature. 27; 486 (7404): 465–6.

Abraham EP, Chain E. 1940. An enzyme from bacteria able to destroy penicillin. Reviews of Infectious Diseases. 10: 677–678.

Adewole DI, Kim IH, Nyachoti CM. 2016. Gut health of pigs: challenge models and re-sponse criteria with a critical analysis of the effectiveness of selected feed additives – a re-view. Asian Australas. J. Anim. Sci. 29, 7: 909–924.

Alcalde-Sanz L, Gawlik BM. 2017. Minimum quality requirements for water reuse in agricultural irrigation and aquifer recharge - towards a legal instrument on water reuse at EU level. Publications Office of the European Union, Luxembourg. 28962.

Ali H, Khan E, Anwar Sajad M. 2013. Phytoremediation of heavy metals – concepts and applications. Chemosphere. 91: 869–881.

Ali S, Abbas Z, Rizwan M, Zaheer IE, et al. 2020. Application of floating aquatic plants in phytoremediation of heavy metals polluted water: a review. Sustainability. 12, 1927.

Almeida CMR, Santos F, Ferreira AC, Gomes CR, Basto MC, Mucha AP. 2016. Constructed wetlands for the removal of metals from livestock wastewater – can the presence of veterinary antibiotics affect removals? Ecotoxicol. Environ. Saf. 137, 143–148.

AlSheikh HMA, Sultan I, Kumar V, Rather IA, Al-Sheikh H, Tasleem Jan A, et al. 2020. Plant-Based phytochemicals as possible alternative to antibiotics in combating bacterial drug resistance. Antibiotics (Basel). 4;9 (8): 480.

Ankri S, Mirelman D. 1999. Antimicrobial properties of allicin from garlic. Microbes Infect 1:125–129.

Apajalahti J, Kettunen A, Graham H. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to chickens. World Poult Sci J. 52, 223–232.

Arango Duque G, Descoteaux A. 2014. Macrophage cytokines: involvement in immunity and infectious diseases. Front. Immunol. 5, 491.

Baydar NG, Özkan G, Sagdiç O. 2004. Total phenolic contents and antibacterial activities of grape (*Vitis vinifera L.*) extracts. Food Control. 15: 335–339.

Beattie JH, Kwun IS. 2004. Is zinc deficiency a risk factor in atherosclerosis? British Journal of Nutrition. 91, 177–181.

Bedford MR, Cowieson AJ. 2012. Exogenous enzymes and their effects on intestinal microbiology. *Anim Feed Sci Technol.* 173, 76–85.

Bee G, Silacci P, Ampuero-Kragten S, Candek-Potokar M, et al. 2017. Hydrolysable tannin-based diet rich in gallotannins has a minimal impact on pig performance but significantly reduces salivary and bulbourethral gland size. Animal. 11, 1617–1625.

Berners-Lee M, Kennelly C, Watson R, Hewitt CN. 2018. Current global food production is sufficient to meet human nutritional needs in 2050 provided there is radical societal adaptation. Elem Sci Anth, 6: 52.

Bhargava A, Carmona FF, Bhargava M, Srivastava S. 2012. Approaches for enhanced phytoextraction of heavy metals. J. Environ. Manage. 105: 103–120.

Biaga G, Cipollini I, Paulicks BR, Roth FX. 2010. Effect of tannins on growth performance and intestinal ecosystem in weaned piglets. Arch. Anim. Nutr. 64, 2: 121–135.

Bindelle J, Pieper R, Leterme P, Rossnagel B, Van Kessel AG. 2010. Changes in intestinal microbial ecophysiology as related to the carbohydrate composition of barleys and oats culti-vars in an *in vitro* model of the pig gastrointestinal tract. Livestock Science. 133, 151–153.

Bolan NS, Khan MA, Donaldson J, Adriano DC, Matthew C. 2003. Distribution and bioavailability of copper in farm effluent. Sci. Total. Environ. 309: 225–236.

Bordet E, Maisonnasse P, Renson P, Bouguyon E, Crisci E, Tiret M, et al. 2018. Porcine alveolar macrophage-like cells are pro-inflammatory pulmonary intravascular macrophages that produce large titers of porcine reproductive and respiratory syndrome virus. Scientific reports. 8(1), 10172.

Bricknell I, Dalmo RA. 2005. The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immunol. 19, 457–472.

Brus M, Bavdek SV, Skok J, Škorjanc D. 2013a. Impact of supplementing pig diet with tannins on histological characteristics of small intestine and growth performance of fattening pigs. Acta Agric. Slov. Suppl. 4, 135–138.

Brus M, Dolinšek J, Cencič A, Škorjanc D. 2013b. Effect of chestnut (*Castanea sativa Mill.*) wood tannins and organic acids on growth performance and faecal microbiota of pigs from 23 to 127 days of age. Bulg. J. Agric. Sci. 19, 4: 841–847.

Brus M, Gradišnik L, Trapečar M, Škorjanc D, Frangež R. 2018. Beneficial effects of water-soluble chestnut (*Castanea sativa Mill.*) tannin extract on chicken small intestinal epithelial cell culture. Poultry science. 97(4): 1271–1282.

Burt SA, van der Zee R, Koets AP, de Graaff AM, van Knapen F, Gaastra W, et al. 2007. Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157: H7. Appl Environ Microbiol 73:4484–4490.

Butaye P, Devriese LA, Haesebrouck F. 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. Clin Microbiol Rev. 16(2): 175–88.

Candek-Potokar M, Skrlep M, Lukac N, Zamaratskaia G, Povse M, Bolta S, et al. 2015. Hydrolysable tannin fed to entire male pigs affects intestinal production, tissue deposition and hepatic clearance of skatole. Vet. J. 204, 162–167.

Cappai MG, Wolf P, Pinna W, Kamphues J. 2012. Dose-response relationship between dietary polyphenols from acorns and parotid gland hypertrophy in pigs. Food. Nutr. Sci. 3, 1261.

Carson CF, Mee BJ, Riley TV. 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrob Agents Chemother 46:1914–1920.

Cereijido M, Contreras RG, Flores-Benítez D, Flores-Maldonado C, Larre I, Ruiz A, et al. 2007. New diseases derived or associated with the tight junction. Med Res Arch. 38, 465–478.

Chandra R, Yadav S. 2010. Potential of *Typha angustifolia* for phytoremediation of heavy metals from aqueous solution of phenol and melanoidin. Ecol. Eng. 1277–1284.

Chardon X, Rigolot C, Baratte C, Espagnol S, Raison C, Martin-Clouaire R, et al. 2012. MELODIE: a whole-farm model to study the dynamics of nutrients in dairy and pig farms with crops. Animal. 6:10, 1711–1721.

Chelakkot C, Ghim J, Ryu SH. 2018. Mechanisms regulating intestinal integrity and its pathological implications. Exp Mol Med. 50, 103.

Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front Microbiol. 5, 69–83.

Commission Regulation (EU). 2016. Concerning the authorization of Zinc acetate dihydrate, Zinc chloride anhydrous, Zinc oxide (…) as feed additive for all animal species. Off J. L: 182/7. 1095.

Cosgrove SE, Carmeli Y. 2003. The impact of antimicrobial resistance on health and economic out-comes. Clin. Infect. Dis. 36: 1433–37.

Cousins RJ, Blanchard RK, Moore JB, Cui L, Green CL, Liuzzi JP, et al. 2003. Regulation of zinc metabolism and genomic outcomes. Journal of Nutrition. 133, 1521–1526.

Cousins RJ. 1996. Zinc. In: Filer LJ, Ziegler EE. (eds) Present Knowledge in Nutrition, 7th edition. International Life Science Institute–Nutrition Foundation, Washington, DC.

Dai SY, Jones B, Lee K-M, Li W, Post L, Herrman TJ. 2016. Heavy metal contamination of animal feed in Texas. Drug. Inf. J. 01: 21–32.

Dalvi AA, Bhalerao SA. 2013. Response of plants towards heavy metal toxicity: an overview of avoidance, tolerance and uptake mechanism. Ann. Plant Sci. 2, 362–368.

Davis A, Sharp J. 2020. Rethinking one health: emergent human, animal and environmental assemblages. Soc. Sci. Med. 258: 113093.

Dewey CE, Cox BD, Straw BE, Bush EJ, Hurd HS. 1997. Associations between off-label feed-additives and farm size, veterinary consultant use, and animal age. Prev. Vet. Med. 31: 133–146.

Dhifi W, Bellili S, Jazi S, Bahloul N, Mnif W. 2016. Essential oils' chemical characterization and investigation of some biological activities: a critical review. Medicines (Basel, Switzerland). 3(4), 25.

Domeneghini C, Di Giancamillo A, Arrighi S, Bosi G. 2006. Gut-trophic feed additives and their effects upon the gut structure and intestinal metabolism. State of the art in the pig, and perspectives towards humans. Histol. Histopathol. 21:273–283.

Dundar E, Olgun EG, Isiksoy S, Kurkcuoglu M, Baser KHC, Bal C. 2008. The efects of intra-rectal and intra-peritoneal application of *Origanum onites* L. essential oil on 2,4,6-trinitrobenzenesulfonic acid-induced colitis in the rat. Exp. Toxicol. Pathol. 59: 399–408.

European Food Safety Authority (EFSA). 2017. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015, EFSA Journal. 15(2): 4694.

European Medicine Agency (EMA). 2017. Questions and answers on veterinary medicinal products containing Zinc Oxide to be administered orally to food-producing species. N˚ 394961.

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

European Parliament and the Council. 2003. Regulation 1831/2003 on additives for use in animal nutrition. Off J. L: 268/29.

FAO. 2011. The state of the world's land and water resources for food and agriculture (SOLAW) – Managing systems at risk. Rome and Earthscan, London.

FAO. 2020. World Food and Agriculture – Statistical Yearbook 2020. Rome.

Favas PJC, Pratas J, Varun M, D'Souza R, Paul MS. 2014. Phytoremediation of soils contaminated with metals and metalloids at mining areas: potential of native flora. InTech.

Gadde U, Kim WH, Oh ST, Lillehoj HS. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Anim Health Res Rev. 18:26–45.

Garcia SN, Osburn BI, Jay-Russell MT. 2020. One health for food safety, food security, and sustainable food production. Front. Sustain. Food Syst. 4(2), 1–9.

Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev. 17, 259–275.

Girard M, Hu D, Pradervand N, Neuenschwander S, Bee G. 2019. Chestnut extract but not sodium salicylate decrease the severity of diarrhea and enterotoxigenic *Escherichia coli* F4 shedding in artificially infected piglets. BioRxiv. 15(2), 0214267.

Girard M, Thanner S, Pradervand N, Hu D, Ollagnier C, Bee G. 2018. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: efficacy on an experimental ETEC F4 model. Plos one. 13,5: 0197878.

Giromini C, Rebucci R, Fusi E, Rossi L, Saccone F, Baldi A, 2016. Cytotoxicity, apoptosis, DNA damage and methylation in mammary and kidney epithelial cell lines exposed to ochratoxin. Cell. Biol. Toxicol. 32(3): 249–258.

Govind P, Madhuri S. 2014. Heavy metals causing toxicity in animals and fishes. Res. J. Animal, Veterinary and Fishery Sci. 2(2): 17–23.

Gresse R, Chaucheyras-Durand F, Fleury MA, van de Wiele T, Forano E, Blanquet-Diot S. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol. 25: 851–73.

Gul S, Naz A, Fareed I, Khan A, Irshad M. 2015. Speciation of heavy metals during co-composting of livestock manure. Pol. J. Chem. Tech. 17, 3: 19–23.

Hao R, Li Q, Zhao J, Li H, Wang W, Gao J. 2015. Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets. Livest. Sci. 178, 237–242.

Hasan K, Cheng Y, Kanwar MK, Chu XY, Ahammed GJ, Qi ZY. 2017. Responses of plant proteins to heavy metal stress – a review. Front. Plant Sci. 8, 1492.

Hazra M, Avishek K, Pathak G. 2015. Phytoremedial potential of *Typha latifolia, Eichornia crassipes* and *Monochoria hastata* found in contaminated water bodies across Ranchi City (India). Int. J. Phytoremediation. 835–840.

Hejna M, Gottardo D, Baldi A, Dell'Orto V, Cheli F, Zaninelli M, et al. 2018. Review: nutritional ecology of heavy metals. Animal. 1–15.

Hejna M, Moscatelli A, Onelli E, Baldi A, Pilu S, Rossi L. 2019. Evaluation of concentration of heavy metals in animal rearing system. Ital. J. Anim. Sci. 18: 1372–1384.

Hejna M, Moscatelli A, Stroppa N, Onelli E, Pilu S, Baldi A, et al. 2020. Bioaccumulation of heavy metals from wastewater through *Typha Latifolia* and *Thelypteris palustris* phytoremediation system. Chemosphere. 241, 125018.

Hogberg A, Lindberg JE. 2004. Influence of cereal non-starch polysaccharides on diges-tion site and gut environment in growing pigs. Livestock Production Science. 87, 121–130.

Huang Q, Liu X, Zhao G, Hu T, Wang Y. 2018. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. Anim. Nutr. 4, 137–150.

Huang Y, Xiao D, Burton-Freeman B, Edirisinghe I. 2016. Chemical changes of bioactive phytochemicals during thermal processing. Reference Module in Food Science.

Hysa E, Kruja A, Rehman NU, Laurenti R. 2020. Circular economy innovation and environmental sustainability impact on economic growth: an integrated model for sustainable development. Sustainability. 12, 4831.

Islam MA, Pröll M, Hölker M, Tholen E, Tesfaye D. 2013. Alveolar macrophage phagocytic activity is enhanced with LPS priming, and combined stimulation of LPS and lipoteichoic acid synergistically induce pro-inflammatory cytokines in pigs. Innate. Immun. 19(6): 631–643.

Jakubus M, Dach J, Starmans D. 2013. Biovailability of copper and zinc in pig and cattle slurries. Fresenius Environ. Bull. 22 (4): 995–1002.

Jensen BB. 1998. The impact of feed additives on the microbial ecology of young pigs. J. Anim. Feed Sci. 7:45–64.

Ji F, McGlone J, Kim S. 2006. Effects of dietary humic substances on pig growth performance, carcass characteristics, and ammonia emission. J. Anim. Sci. 84, 2482–2490.

Jindal BAK, Pandya MK, Khan, MID. 2015. Antimicrobial resistance: a public health challenge. Med. J. Armed Forces India. 71: 178–181.

Kadlec RH, Knight RL, Vymazal J, Brix H, Cooper P, Haberl R. 2000. Constructed wetlands for pollution control: processes, performance, design and operation. Scientific and Technical Report, IWA Publishing, London, UK, 8, 1–15.

Kagnoff MF. 2014. The intestinal epithelium is an integral component of a communica-tions

Kahrstrom CT, Pariente N, Weiss U. 2016. Intestinal microbiota in health and disease. Nature. 535, 47.

Kalemba D, Synowiec A. 2020. Agrobiological interactions of essential oils of two menthol mints: *Mentha piperita* and *Mentha arvensis*. Molecules. 25(1): 59.

Kesavan PC, Swaminathan MS. 2008. Strategies and models for agricultural sustainability in developing Asian countries. Phil. Trans. R. Soc. B. 363, 877–891.

Khanbabaee K, van Ree T. 2001. Tannins: classification and definition. Nat Prod Rep. 18,6: 641–649.

Kim DK, Lillehoj HS, Lee SH, Jang SI, Bravo D. 2010. High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or Capsicum oleoresin. Poult Sci. 89:68–81.

Kim K, He Y, Xiong X, Ehrlich A, Li X, Raybould H, et al. 2019. Dietary supplementation of *Bacillus subtilis* influenced intestinal health of weaned pigs experimentally infected with a pathogenic *E. coli.* J Anim. Sci. Biotechno. 10: 52.

Klink A, Macioł A, Wisłocka M, Krawczyk J. 2013. Metal accumulation and distribution in the organs of *Typha latifolia L.* (cattail) and their potential use in bioindication. Limnologica. 164–168.

Knight RL, Payne VWE, Borer RE, Clarke RA, Pries JH. 2000. Constructed wetlands for livestock wastewater management. Ecol. Eng. 15, 41–55.

Konstantinov SR, Awati A, Smidt H, Williams BA, Akkermans ADL, de Vos WM. 2004. Specific response of a novel and abundant Lactobacillus amylovorus-like phylotype to dietary prebiotics in the guts of weaning piglets. Applied and Environmental Microbiology. 70, 3821–3830.

Krzeslowska, M. 2011. The wall cell in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy. Acta Physiol. Plant. 33, 35–51.

Lambert RJW, Skandamis PN, Coote PJ, Nychas G. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 91:453–462.

Landers TF, Cohen B, Wittum TE, Larson EL. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep. 127(1):4–22.

Lee CY, Lee CC, Lee FY, Tseng SK, Liao CJ. 2004. Performance of subsurface flow constructed wetland taking pre-treated swine effluent under heavy loads. Bioresource Technol. 92, 173–179.

Li Y, Wu Z, Liu K, Qi P, Xu J, Wei J, et al. 2017. Proteomic analysis of the secretome of porcine alveolar macrophages infected with porcine reproductive and respiratory syndrome virus. Proteomics. 17: (21).

Liu Y, Song M, Che T, Bravo D, Pettigrew J. 2012. Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages *in vitro*. J. Anim. Sci. 90: 2774–2783.

Lopez-Alonso M, Garcia-Vaquero M, Benedito JL, Castillo C, Miranda M. 2012. Trace mineral status and toxic metal accumulation in extensive and intensive pigs in NW Spain. Livest. Sci. 47–53.

Luo L, Maa Y, Zhang S, Wei D, Zhu Y-G. 2009. An inventory of trace element inputs to agricultural soils in China. J Environ. Manage. 90: 2524–2530.

Lyubenova L, Schröder P. 2011. Plants for waste water treatment – effects of heavy metals on the detoxification system of *Typha latifolia*. Bioresour. Technol. 102: 996–1004.

Małachowska-Jutsz A, Gnida A. 2015. Mechanisms of stress avoidance and tolerance by plants used in phytoremediation of heavy metals. Arch. Environ. Prot. 41, 104–114.

Manousaki E, Kalogerakis N. 2009. Phytoextraction of Pb and Cd by the mediterranean saltbush (*Atriplex halimus L.*): metal uptake in relation to salinity. Environ. Sci. Pollut. Res. Int. 16(7): 844–54.

Maric M, Antonijevic M, Alagic S. 2013. The investigation of the possibility for using some wild and cultivated plants as hyperaccumulators of heavy metals from contaminated soil. Environ. Sci. Pollut. Res. 20: 1181–1188.

Meers E, Rousseau DPL, Blomme N, Lesage E, Du Laing G, Tack FMG,et al. 2005. Tertiary treatment of the liquid fraction of pig manure with *Phragmites australis*. Water Air Soil Pollut. 160, 15–26.

Memon AR, Schröder P. 2009. Implications of metal accumulation mechanisms to phytoremediation. Environ. Sci. Pollut. R. 16.

Milanov DS, Ljubojević DB, Čabarkapa IS, Aleksić NZ. 2016. Impact of antibiotics as growth promoters on bacterial resistance. Food and Feed Research, 43 (2), 83–92.

Millet S, Maertens L. 2011. The European ban on antibiotic growth pro-moters in animal feed: from challenges to opportunities.Vet. J. 187, 143–144.

Miransari M. 2011. Hyperaccumulators, arbuscular mycorrhizal fungi and stress of heavy metals, Biotec. Adv. 29, 645–653.

Moeser AJ, Pohl CS, Rajput M. 2017. Weaning stress and gastrointestinal barrier development: implications for lifelong gut health in pigs. Anim Nutr. 3(4): 3–21.

Montagne L, Pluske JR, Hampson DJ. 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Animal Feed Science and Technology. 108, 95–117.

Moral R, Moreno-Caselles J, Perez-Murcia MD, Perez-Espinosa A, Rufete B, Paredes C. 2005. Characterisation of the organic matter pool in manures. Bioresour Technol. 153–158.

Moustafa K, Cross J, Gasim S. 2018. Food and starvation: is Earth able to feed its growing population? Int. J. Food Sci. 69 (4): 385–388.

Munita JM, Arias CA. 2016. Mechanisms of antibiotic resistance. Microbiology Spectrum. 4: 10.1128.

Murphy K, Weaver C, 2017. Janeway's Immunobiology, 9th edition. Garland Science.

Nicholson FA, Smith SR, Alloway BJ, Carlton-Smith C, Chambers BJ. 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. Sci. Total Environ. 311: 205–219.

O'Neill J et al. 2015. Antimicrobials in agriculture and the environment. Reducing unneccesary use and waste. Review on antimicrobial resistance.

OECD/FAO. 2020. OECD-FAO Agricultural Outlook 2020-2029, OECD Publishing, Paris/ FAO, Rome.

Omonijo FA, Ni L, Gong J, Wang Q, Lahaye L, Yang C. 2018. Essential oils as alternatives to antibiotics in swine production. Animal nutrition. 4(2), 126–136.

Onelli E, Moscatelli A, Gagliardi A, Zaninelli M, Bini L, Baldi A. 2017. Retarded germination of *Nicotiana tabacum* seeds following insertion of exogenous DNA mimics the seed persistent behavior. PloS one. 12(12): 0117–29.

Park YJ, Baek SA, Choi Y, Kim JK, Park SU. 2019. Metabolic profiling of nine *Mentha* species and prediction of their antioxidant properties using chemometrics. Molecules. 24(2): 258.

Parys AV, Boyen F, Dewulf J, Haesebrouck F, Pasmans F. 2010. The use of tannins to control *Salmonella typhimurium* infections in pigs. Zoonoses Public Health. 57(6): 423–8.

Peralta-Videaa JR, Gardea-Torresdeya JL, Gomezc E, Tiemanna KJ, Parsonsa JG, Carrillod G. 2002. Effect of mixed cadmium, copper, nickel and zinc at different pHs upon alfalfa growth and heavy metal uptake. Environ. Pollut. 119: 291–301.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53: 28–52.

Pie S, Lallees JP, Blazy F, Laffitte J, Seve B, Oswald IP. 2004. Weaning is associated with an upregulation of expression if inflammatory cytokines in the intestine of piglets. J Nutr. 134: 641–7.

Pluske JR, Turpin DL, Kim J-Ch. 2018. Gastrointestinal tract (gut) health in the young pig. Animal Nutrition. 4: 187–196.

Polen T, Voia OS. 2015. Copper effect of feed supplementation on growth performance in fattening pigs. Anim. Sci. J. 48(2): 28–30.

Pretty J. 2008. Agricultural sustainability: concepts, principles and evidence. Philos. Trans. R Soc. Lond. B Biol. Sci. 363(1491): 447–465.

Raubenheimer D, Simpson SJ, Mayntz D. 2009. Nutrition, ecology and nutritional ecology: toward an integrated framework. Functional Ecology. 23, 4–16.

Reggi S, Giromini C, Dell'Anno M, Baldi A, Rebucci R, Rossi L. 2020. *In vitro* digestion of chestnut and quebracho tannin extracts: Antimicrobial effect, antioxidant capacity and cytomodulatory activity in swine intestinal IPEC-J2 cells. Animals. 10, 195.

Reimann C, Birke M, Demetriades A, Filzmoser P, O'Connor P. 2014. Chemistry of Europe's agricultural soils, Part a: Methodology and interpretation of the GEMAS data set, Geologisches Jahrbuch Reihe B, Band B 102. Schweizerbart Science Publishers, Stuttgart: 523.

Reimann C, Fabian, Birke M, Filzmoser P, Demetriades A, Négrel P et al. 2018. GEMAS: Establishing geochemical background and threshold for 53 chemical elements in European agricultural soil. Applied Geochemistry. 88, Part B, 302–318.

Rescigno M. 2011. The intestinal epithelial barrier in the control of homeostasis and immunity. Trends Immunol. 32, 256–264.

Rossi L, Dell'Orto V, Vagni S, Sala V, Reggi S, Baldi A. 2014a. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. Vet. Res. Commun. 38(1): 39–49.

Rossi L, Di Giancamillo A, Reggi S, Domeneghini C, Baldi A, Sala V, et al. 2013. Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. J. Vet. Sci. 14(3): 263–270.

Rossi L, Pinotti L, Agazzi A, Dell'Orto V, Baldi A. 2014b. Plant bioreactors for the antigenic hook-associated flgK protein expression. Ital. J. Anim. Sci. 13(1): 23–29.

Rousham EK, Unicomb L, Islam MA. 2018. Human, animal and environmental contributors to antibiotic resistance in low-resource settings: integrating behavioural, epidemiological and One Health approaches. Proc Biol Sci. 285 (1876): 20180332.

Salem ZB, Laffray X, Al-Ashoor A, Ayadi H, Aleya L. 2017. Metals and metalloid bioconcentrations in the tissues of *Typha latifolia* grown in the four interconnected ponds of a domestic landfill site. J. Environ. Sci. 56–68.

Sales J. 2013. Effects of pharmacological concentrations of dietary Zinc Oxide on growth of post-weaning pigs: a meta-analysis. Biol. Trace Elem. Res. 152: 343–349.

Santos D, Luzio RVA, Félix L. 2018. Chapter five – zebrafish early life stages for toxicological screening: insights from molecular and biochemical markers. Advances in Molecular Toxicology. 12: 151–179.

Seal BS, Lillehoj HS, Donovan DM, Gay CG. 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim. Health Res. Rev. 14 78–87.

Seth CS. 2012. A review on mechanisms of plant tolerance and role of transgenic plants in environmental clean-up. Bot. Rev. 78, 32–62.

Shabani N, Sayadi MH. 2012. Evaluation of heavy metals accumulation by two emergent macrophytes from the polluted soil: an experimental study. Environmentalist. 32, 91–98.

Steendam CA, Tamminga S, Boer H, de Jong EJ, Visser GH, Verstegen MW. 2004. Ileal endogenous nitrogen recovery is increased and its amino acid pattern is altered in pigs fed quebracho extract. J. Nutr. 134, 11: 3076–3082.

Stefanakis A, Akratos C, Tsihrintzis V. 2014. Constructed wetlands classification. In: vertical flow constructed wetlands, Publisher: Elsevier. 2014, 17–25.

Stein HH, Kil DY. 2006. Reduced use of antibiotic growth promoters in diets fed to weanling piglets: dietary tools, part 2. Anim. Biotech. 17:217–231.

Stokstad ELR, Jukes TH. 1950. Further observations on the"animal protein factor". Proc. Soc. Exp. Biology and Medicine. 73: 523–528.

Stroppa N, Onelli E, Hejna M, Rossi L, Gagliardi A, Bini L, et al. 2020. *Typha latifolia* and *Thelypteris palustris* behavior in a pilot system for the refinement of livestock wastewaters: a case of study. Chemosphere. 240, 124915.

Suleiman N, Ibitoye EB, Jimoh AA, Sani ZA. 2015. Assessment of heavy metals in chicken feeds available in Sokoto, Nigeria. Sokoto Journal of Veterinary Sciences. 13(1): 17–21.

Suttle NF. 2010. Mineral Nutrition of Livestock, 4th Edition. Cabi, Oxfordshire, UK.

Swift S, Downie JA, Whitehead NA, Barnard AM, Salmond GP, Williams P. 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Adv Microb Physiol. 45, 199–270.

Tang KL, Caffrey NP, Nóbrega DB, Cork SC, Ronksley PE, Barkema HW, et al. 2017. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. Lancet Planet Health. 1(8): 316–327.

Thangavel P, Subbhuraam C. 2004. Phytoextraction: role of hyperaccumulators in metal contaminated soils. Proc. Natl. Acad. Sci. 70: 109–130.

Trckova M, Lorencova A, Babak V, Neca J, Ciganek M. 2017. Effects of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on the health, oxidative stress status and fatty acid profile in weaned piglets. Veterinární medicína. 62(1), 16–28.

UN. 2017. Department of Economic and Social Affairs (DESA) population division world population prospects: the 2017 revision, key findings and advance tables. ESA/P/WP/248.

Upton M. 2004. The role of livestock in economic development and poverty reduction. Food and Agriculture Organization of the United Nations, Pro-Poor Livestock Policy Initiative, PPLPI Working Papers.

Van Den Bogaard AE, Ruinsma NB, Tobberingh EES. 2000. The effect of banning avoparcin on VRE carriage in the Netherlands (Five Abattoirs) and Sweden. Journal of Anti-microbial Chemotherapy. 46:146–148.

Velten S, Leventon J, Jager N, Newig J. 2015. What is sustainable agriculture? A systematic review. Sustainability. 7, 7833–7865.

Walk CL, Wilcock P and Magowan E. 2015. Evaluation of the effects of pharmacological zinc oxide and phosphorus source on weaned piglet growth performance, plasma minerals and mineral digestibility. Animal. 9: 1145–1152.

Wang L, Hu S, Liu Q, Li Y, Xu L, Zhang Z, et al. 2017. Porcine alveolar macrophage polarization is involved in inhibition of porcine reproductive and respiratory syndrome virus (PRRSV) replication. J. Vet. Med. Sci. 79: 1906–1915.

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

Wittebole X, De Roock S, Opal SM. 2014. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. Virulence 5, 226–235.

World Health Organization (WHO). 2000. Global principles for the containment of antimicrobial resistance in animals intended for food: report of a WHO consultation with the participation of the Food and Agriculture Organization of the United Nations and the Office International des Epizooties, Geneva, Switzerland. 5–9.

World Health Organization (WHO). 2015. Global action plan on antimicrobial resistance. Geneva: World Health Organization.

Wu Z, Tan B, Liu Y, Dunn J, Martorell Guerola P, et al. 2019. Chemical composition and antioxidant properties of essential oils from peppermint, native spearmint and scotch spearmint. Molecules. 24(15): 2825.

Yadav SK. 2010. Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South Afr. J. Bot. 76, 167–179.

Yan A, Wang Y, Tan SN, Mohd-Yusof ML Ghosh S, Chen Z. 2020. Phytoremediation: a promising approach for revegetation of heavy metal-polluted land. Front. Plant Sci. 11, 359.

Zhang F, Li Y, Yang M, Li W. 2012. Content of heavy metals in animal feeds and manures from farms of different scales in northeast China. Int. J. Environ. Res. Public. Health. 9: 2658–2668.

CHAPTER 2

Aim

Agriculture raises a number of questions related to (i) the importance of one health approach where human, animal and environmental health should be systemically linked, (ii) the environmental impact of food-producing animals and crops production systems (iii), the moving towards the sustainable animal production systems and importance of nutritional ecology in enhancing animal performance and health. Moreover, considering the need to reduce of the use of antibiotics and wide application of high doses of zinc and copper salts in the form of premix, which in the last decade was the first adopted alternative against in-feed antibiotics, the main aim of the present work was to develop integrative strategies to reduce use of antibiotics and zinc and copper in swine rearing system by reducing their input and output (Figure 8).

Thus, the first aim was to review the current scenario and focused on the nutritional ecology of heavy metals, legal regulations related to heavy metals' contamination established in the European Union and on the major criticisms related to the heavy metal content in animal feeds, manure, soil and animal-origin products (first study) as a basis for developing the effective plant-based approaches to the reduction of heavy metal pollution from livestock. Then, the second aim was to evaluate the concentration of heavy metals and mineral nutrients from feed, water, and faeces in modern swine and cattle rearing systems in Italy, where swine production is a major part of intensive animal production (second study).

Considering the need for alternatives to in-feed antibiotics and reducing the dietary input of Zn and Cu, the innovative effective plant-based additives and phytochemicals were tested in order to confirm their antimicrobial, anti-inflammatory activities and to improve gut health of weaned pigs. Thus, the third aim was (i) to evaluate the *in vivo* effect of chestnut and quebracho tannins in order to establish if the inclusion can induce a positive effect on weaned piglets, (ii) to evaluate the *in vivo* effect of leonardite included at 0.25% on the principal metabolic parameters and growth of weaned piglets (third and fourth studies) and (iii) to measure the *in vitro* anti-inflammatory effects of peppermint oil and spearmint oil with porcine alveolar macrophages as host immune responses (preliminary data of fifth experiment).

Moreover, accounting for the release of high amount of heavy metals by food-producing animals through faeces, the fourth aim of this study was to assess the ability of two aquatic species, *Typha latifolia* (Broadleaf cattail) and *Thelypteris palustris* (Marsh fern), to remove Zn and Cu from contaminated livestock wastewater (sixth study).

Figure 8. The scheme of chapters and aim discussed in the thesis.

Chapter 1
Scientific background

Chapter 2
Aim

Develop integrative strategies to reduce use of antibiotics and zinc and copper in swine rearing system by reducing their input and output.

Chapter 3 **An overview about research topic**	Review: Nutritional ecology of heavy metals. Hejna M, et al. 2018. Animal. 1-15. Evaluation of concentration of heavy metals in animal rearing system. Hejna M, et al. 2019. It. J. Anim. Sc. 18 (1), 1372–1384.
Chapter 4 **Reducing input**	Evaluation of dietary administration of chestnut and quebracho tannins on growth, serum metabolites and faecal parameters of weaned piglets. Caprarulo V, Hejna M, et al. 2020. Animals. 10, 1945. Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets. Dell'Anno M, Hejna M, et al. 2020. Anim. Feed Sci. Technol. 266, 114519. Mint oils and their *in vitro* anti-inflammatory effects tested in porcine alveolar macrophages. Hejna M, et al. American Society of Animal Science: 2020 Virtual Annual Meeting and Trade Show in Madison, Wisconsin (USA).
Chapter 5 **Reducing output**	Bioaccumulation of heavy metals from wastewater through a *Typha latifolia* and *Thelypteris palustris* phytoremediation system. Hejna M, et al. 2020. Chemosphere. 241, 125018.

Chapter 6
General discussion and conclusion

CHAPTER 3

Brief introduction of the scientific works of the importance of heavy metals in animal rearing system

The role of nutrition in food-producing animals is one of the important subject and the content of essential and non-essential heavy metals in animal feed and manure is a base for developing effective approaches of reduction of heavy metal pollution from livestock wastewater in order to maintain sustainable production in accordance with one health approach. This chapter is therefore focused on the overview about main challenges related to (i) heavy metals and human health, (ii) the legislative regulations adapted in the European Union, (iii) heavy metals in feed and food chain, (iv) mineral nutrition in livestock, (v) heavy metals as essential and non-essential elements in feed and livestock manure, (vi) heavy metal content in agricultural soil and (vii) strategies to control heavy metal pollution. Moreover, this chapter focused on the evaluation of concentration of heavy metals from feed, faeces and water samples in animal rearing system in Lombardy region (Italy) from different phases of swine production (gestation, farrowing, weaning and finishing) and cattle production (calves and lactation) with special attention on the zinc and copper elements.

Review: Nutritional ecology of heavy metals

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3.1. Abstract

The aim of this review is to focus the attention on the nutrition ecology of the heavy metals and on the major criticisms related to the heavy metals content in animal feeds, manure, soil and animal origin products. Heavy metals are metallic elements that have a high density that have progressively accumulated in the food chain with negative effects for human health. Some metals are essential (Fe, I, Co, Zn, Cu, Mn, Mo, Se) to maintain various physiological functions and are usually added as nutritional additives in animal feed. Other metals (As, Cd, F, Pb, Hg) have no established biological functions and are considered as contaminants/undesirable substances. The European Union (EU) adopted several measures in order to control their presence in the environment, as a result of human activities such as farming, industry or food processing and storage contamination. The control of the animal input could be an effective strategy to reduce human health risks related to the consumption of animal-origin products and the environmental pollution by manure. Different management of raw materials and feed, animal species as well as different legal limits can influence the spread of heavy metals. To set up effective strategies against heavy metals the complex interrelationships in rural processes, the widely variability of farming practices, the soil and climatic conditions must be considered. Innovative and sustainable approaches have discussed for the heavy metal nutrition ecology to control the environmental pollution from livestock-related activities.

3.2. Introduction

Metals are natural constituents of the earth's crust and through natural erosion due to water and wind, they are naturally spread into the environment as powders or leached into rivers. However, these natural processes emit fewer metals into the environment than anthropological activities. The spread of high amounts of these elements in the environment leads to their propagation in the food chain. Heavy metals (e.g. Fe, Co, Cu, Mn, Mo, Se, Zn, Cr and Cd, Hg, Pb, As) are metallic elements that have a high density compared to water and are present in various matrices in traces. Their heaviness and toxicity are interrelated, as heavy metals are able to induce toxicity at low doses (Bhargava et al., 2012; Govind and Madhuri, 2014; Dai et al., 2016; Giromini et al., 2016).

Some metals are essential to maintain various biochemical and physiological functions in humans, animals and plants. The nutritional requirements of these trace elements, such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se) and zinc (Zn) are generally low and they are called microelements. They are present in various matrices, although with a different bioavailability, in trace concentrations (ppb or ppm) (Hambidge, 2003). Essential trace elements are usually added as nutritional additives in animal feed to promote health, and to optimize production (EU Reg. 1881/2006). However, excessive exposure with higher concentration of these elements has been linked with cellular or systemic disorders and could represent a source of pollution (Rossi et al., 2014b).

Other metals (e.g. As, Cd, Pb, Hg) have no established biological functions and are considered as contaminants and undesirable substances in animal feed (Reg. 2002/32/EC). Moreover, As, Cd, Cr, Pb and Hg which are a prior hazard to public health, present a high toxicity because they can induce organ damage, even at lower exposure levels (Table 3).

Table 3. Heavy metals in nutrition **(**Theron et al., 2012; Govind and Madhuri, 2014).

Heavy metals	
Essential elements (authorized in animal nutrition*)	**Nonessential elements (undesirable**)**
Co (cobalt)	As (arsenic)
Cr (chromium)	Cd (cadmium)
Cu (copper)	Hg (mercury)
Fe (iron)	Pb (lead)
Mn (manganese)	
Mo (molybdenum)	
Ni (nickel)	
Se (selenium)	
Zn (zinc)	

* additives authorized in animal nutrition according to EC N1831/2003;
** undesirable elements according to 2002/32/EC.

The toxicity of heavy metals, whether essential or not, depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of the exposed individuals (Tchounwou et al., 2012). They have different effects in relation to the dose and the time of consumption: acute poisoning for high doses in a short period and chronic poisoning or bioaccumulation for reduced exposure over a long period. In the long term, the accumulation of heavy metals in soil can lead to a deterioration of agricultural land, eutrophication and the absorption of toxic substances. This could have long-term implications for the quality of agricultural soils, including phytotoxicity at high concentrations, the maintenance of soil microbial processes, and the transfer of zootoxic elements to the human diet due to an increased crop uptake or soil ingestion by grazing livestock (Nicholson et al., 2003).

In the last decade the EU has been promoting the reduction of nutrient and heavy metal pollution of water and soil given that such pollutants are the major cause of eutrophication. The contamination of food and feed with heavy metals has become a serious problem in intensive agriculture.

Moreover, with regard to farming livestock, a global nutritional ecology strategy is needed in order to guarantee both the health status of humans and animals and sustainable productions. The ecology of nutrition is a multidisciplinary approach, mainly focusing on living organisms, the environment and the nutritional basis of the cooperation between organisms (function, mechanism, development) and the environment (biotic and abiotic) (Raubenheimer et al., 2009). Considering the great variety of heavy metals in the environment, it is impossible to avoid the presence of heavy metals in the food chain, and in the environment.

This study focuses on the role of animal production and on the main challenges related to the content of essential and nonessential heavy metals in animal feed and manure as a basis for developing effective approaches to the reduction of heavy metal pollution from livestock.

3.3. Legislative context

The EU authorities have thus adopted various measures to control heavy metals presence in the environment, as a result of human activities such as farming, industry, and food processing and storage contamination. Therefore, reducing heavy metal inputs to the environment and the absolute decoupling are the main focus of EU environmental protection policies given that promote continued economic growth with a reduction in environmental impacts (Jarup, 2003). The EU has established comprehensive regulations on the maximum authorized admissible concentrations of essential (Fe, I, Co, Cu, Mn, Zn, Mo, Se) and

undesirable substances (As, Cd, F, Pb, Hg) (Table 4). Moreover, different maximum inclusion levels of essential trace elements in additives for use in animal nutrition have been set (Table 5). Main aim of those regulations (EC Reg. No 1831/2003; Dir. 2002/32/EC) is to protect feed and food safety, and ultimately human health, and reducing environmental pollution (Fink-Gremmels, 2012).

Table 4. Main EU regulations concerning essential and undesirable substances contamination.

Regulation	Full title and main content	EUR-lex link
Directive 2002/32/EC	Directive of the European Parliament and of the council of 7 May 2002 on undesirable substances in animal feed.	http://data.europa.eu/eli/dir/2002/32/oj
Regulation (EC) N° 1831/2003	Establishes a procedure for authorising the placing on the market and use of feed additives and to lay down rules for the supervision and labelling of feed additives and premixtures.	http://data.europa.eu/eli/dir/2003/1831/oj
Regulation (EC) N° 1881/2006	Represents the main guidelines concerning contaminants in foodstuffs; it sets maximum levels of mycotoxins, metals (Pb, Cd, Hg and Sn), dioxins and PCBs in different food sources.	http://data.europa.eu/eli/reg/2006/1881/oj
Regulation (EC) N° 776/2006	Defines the community reference laboratories responsible for the official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.	http://data.europa.eu/eli/reg/2006/776/oj
Regulation (EC) N° 1754/2006	Lays down detailed rules for the granting of Community financial assistance for the organisation of workshops.	http://data.europa.eu/eli/reg/2006/1754/oj
Regulation (EC) N° 333/2007	Specifies the sampling methods and the methods of analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. It is divided into four parts (A, B, C and D) that deal with the "definitions", "sampling methods", "sample preparation and analysis" and lastly "reporting and interpretation of results" respectively.	http://data.europa.eu/eli/reg/2007/333/oj
Regulation (EC) N° 629/2008	Amends the previous Regulation (EC) No 1881/2006 that sets maximum levels for certain contaminants in foodstuffs. It modifies the maximum levels for lead, cadmium and mercury. Regarding lead, this regulation adds the category of "food supplements" and specifies the species of cultivated mushrooms. For cadmium and mercury, it makes the list of fish species easier to consult.	http://data.europa.eu/eli/reg/2008/629/oj
Regulation (EC) N° 767/2009	Regulation of the European parliament and of the council of 13 July 2009 on the use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 amending the conditions for authorization of a number of additives in feedstuffs belonging to the group of trace elements.	http://data.europa.eu/eli/reg/2009/767/oj

Regulation (EU) N° 836/2011	Amends Regulation (EC) No 333/2007 and changes the title to: "laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs".	http://data.europa.eu/eli/reg/2011/836/oj
Regulation (EU) N° 1275/2013	Amends Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic, cadmium, lead, nitrites, volatile mustard oil and harmful botanical impurities, and reduces the levels of arsenic and lead and modifies the feed materials concerning volatile mustard oil levels.	http://data.europa.eu/eli/reg/2013/1275/oj
Regulation (EU) N° 488/2014	Amends Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs. It now includes different categories such as "Infant formulae"," Processed cereal-based foods and baby foods for infants and young children" and "Specific cocoa and chocolate products".	http://data.europa.eu/eli/reg_impl/2014/448/oj
Regulation (EU) N° 1005/2015	Amends the Regulation (EC) No 1881/2006 modifying the maximum levels of lead in certain foodstuffs such as foods for special medical purposes intended specifically for infants and young children, and also beverages.	http://data.europa.eu/eli/reg/2015/1005/oj
Regulation (EU) N° 1006/2015	Amends the previous Regulation (EC) No 1881/2006 as regards maximum levels of inorganic arsenic and introduces a subsection on arsenic levels in rice and rice products.	http://data.europa.eu/eli/reg/2015/1006/oj
Regulation (EU) N° 582/2016	Amends Regulation (EC) No 333/2007. The main differences concern the title and table 5 regarding the "Performance criteria for methods of analysis for lead, cadmium, mercury, inorganic tin and inorganic arsenic" where the maximum levels of cadmium, mercury, inorganic arsenic and lead are added.	http://data.europa.eu/eli/reg/2016/582/oj

Considering the great variety of heavy metals in the environment and their concentration within different feed production chains, it is impossible to achieve lower levels of contamination than the detection limit for all elements in all products. It is therefore necessary to work at different levels (Aragay et al., 2011). The EU is aware of all these problems and several laws have thus been enacted in order to control all heavy metal pollution, reduce the risk of human exposure in the food chain as well as setting up detection methods to control these contaminants in the food chain.

Table 5. Maximum levels of essential trace elements and undesirable substances in feeds according to different EU legislation (EC N° 1831/2003; 2002/32/EC) and nutritional requirement for different species (according to: NRC, 2012 for pigs and cattle).

Element	EEC N°	Sources	Nutritional requirements	Maximum level mg/kg d.m. of complete diet
Essential trace elements				
Iron (Fe)	**E1**	Carbonate ferrous Ferrous chlorides Ferrous fumarate Iron oxide Ferrous sulfates Ferrous hydrate chelates	Swine: Pigs 5-7 kg: 100 ppm Gestation: 80 ppm Lactation: 80 ppm Cattle: Growing: 50 mg/kg Gestation: 50 mg/kg Early lactation: 50 mg/kg	Swine: Weaning: 250 mg/day Other pigs: 750 mg/kg Cattle: 750 mg/kg
Iodine (I)	**E2**	Calcium iodide hexahydrate Anhydrous calcium iodate Sodium iodide Potassium iodide	Swine: Pigs 5-7 kg: 0,14 ppm Gestation: 0,14 ppm Lactation: 0,14 ppm Cattle: Growing: 0,5 mg/kg Gestation: 0,5 mg/kg Early lactation: 0,5 mg/kg	Swine: Pigs: 10 mg/kg Cattle: Dairy cows: 5 mg/kg Other categories: 10 mg/kg
Cobalt (Co)	**E3**	Acetate tetrahydrate cobalt Carbonate monohydrate cobalt Cobalt chloride hexahydrate Cobalt sulphates	 Cattle: Growing: 0,1 mg/kg Gestation: 0,1 mg/kg Early lactation: 0,1 mg/kg	Pigs: 2 mg/kg Bovine: maximum 1 mg/kg (0,3 mg/kg recommended)
Copper (Cu)	**E4**	Copper acetate monohydrate Carbonate monohydrate copper Cupric chloride dihydrate Copper methionate Cupric oxide Cupric sulphates Copper chelates	Swine: Pigs 5-7 kg: 6 ppm Gestation: 10 ppm Lactation: 20 ppm Cattle: Growing: 10 mg/kg Gestation: 10 mg/kg Early lactation: 10 mg/kg	Swine: Piglets up to 12 weeks: 170 mg/kg Other pigs: 25 mg/kg Cattle: Milking cows: 15 mg/kg Other bovine: 35 mg/kg
Manganese (Mn)	**E5**	Manganous carbonate	Swine: Pigs 5-7 kg: 4 ppm Gestation: 25 ppm Lactation: 25 ppm	All species: 150 mg/kg

		Manganous chloride tetrahydrate Manganese oxide Manganous sulphates Manganese hydrates Manganese chelates	Cattle: Growing: 20 mg/kg Gestation: 40 mg/kg Early lactation: 40 mg/kg	
Zinc (Zn)	**E6**	Zinc lactate hydrates Zinc carbonate Zinc chloride monohydrate Zinc oxide Zinc sulfates Zinc chelates	Swine: Pigs 5-7 kg: 100 ppm Gestation: 100 ppm Lactation: 100 ppm Cattle: Growing: 30 mg/kg Gestation: 30 mg/kg Early lactation: 30 mg/kg	Pigs: 150 mg/kg Cattle: 100 mg/kg
Molybdenum (Mo)	**E7**	Ammonium molybdate Sodium molybdate		All species: 2,5 mg/kg
Selenium (Se)	**E8**	Sodium selenite Sodium selenate Produced by Saccharomyces cerevisiae strains	Pigs 5-7 kg: 0,3 ppm Gestation: 0,15 ppm Lactation: 0,15 ppm Cattle: Growing: 0,1 mg/kg Gestation: 0,1 mg/kg Early lactation: 0,1mg/kg	All species: 0,5 mg/kg
Undesirable substances (and nonessential trace elements)				
Arsenic (As)		Complete feed	-	2 mg/kg
Cadmium (Cd)		Complete feed	-	Cattle, sheep, goats: 1 mg/kg Others: 0,5 mg/kg
Lead (Pb)		Complete feed	-	All species: 5 mg/kg
Mercury (Hg)		Complete feed	-	All species: 0,1 mg/kg

3.4. Heavy metals and human health

Humans and animals can be exposed to heavy metals and trace elements through different routes: the inhalation of air pollutants, consumption of contaminated drinking water, exposure to contaminated soils or industrial waste, and the ingestion of contaminated food, such as vegetables, grains, fruits, fish and shellfish and meat (Duruibe et al., 2007). The main source of exposure differs according to the elements. For example, Cd is present at low levels in most foods, such as whole grain cereals, fruit, root vegetables, meat and fish. The highest levels of

Cd metals are found in the offal (kidney and liver) of mammals and in mussels, oysters and scallops. Heavy metals cause serious impacts on human and animal health including reduced growth and development, cancer, organ damage, nervous system damage, and in extreme cases, death (Table 6) (Thirulogachandar et al., 2014). The International Agency for Research on Cancer (IARC) considered some heavy metals as carcinogenic to humans, based on evidence on human studies. Some metals are particularly toxic to the sensitive, rapidly-developing systems of fetuses, infants, and young children. Pb and Hg in particular, can easily cross the placenta and damage the fetal brain (Food Safety Authority of Ireland, 2009).

Table 6. Effects of different essential heavy metals on human health and their specific food sources.

Trace elements	Effect associated with human health	Source	Reference
Copper (Cu)	Anemia, liver and kidney damage, and stomach and intestinal irritation.	Soil contamination.	Thirulogachandar et al. (2014)
Nickel (Ni)	Long-term exposure causes decreased body weight, heart and liver damage, and skin irritation.	Volcanic activity, industrial and anthropogenic processes (metal-plating industries, combustion of fossil fuels, and nickel mining and dredging).	EFSA (2015)
Iron (Fe)	Anemia, heart disease, cancer, diabetes, choroiditis, retinitis and conjunctivitis.	Meat, whole meal products, potatoes and vegetables.	Thirulogachandar et al. (2014)
Cobalt (Co)	Sterility, hair loss, vomiting, bleeding, diarrhoea, coma and even death.	Soil contamination.	Thirulogachandar et al. 2014
Manganese (Mn)	Languor, sleepiness, weakness, emotional disturbances, spastic gait, recurring leg cramps and paralysis.	Spinach, tea, herbs, grains and rice, soya beans, eggs, nuts, olive oil, green beans and oysters.	Thirulogachandar et al. 2014

Childhood exposure to some metals can result in learning difficulties, memory impairment, damage to the nervous system, and behavioral problems such as aggressiveness and hyperactivity. At higher doses, heavy metals can cause irreversible brain damage (Jaishankar et al., 2014). It is also very important to consider that children receive higher doses of metals from food than adults, since they consume more food in relation to their body weight (Thirulogachandar et al., 2014). The probability that a population will be exposed and harmed could be higher for a metal with a moderate toxicity but which is widespread and thus there is

a higher risk of exposure. The environmental conditions, such as soil contamination, industrial processes and incorrect manufactory procedures, are responsible for high levels of Sn, Cu, Ni and Co.

3.5. Heavy metals in feed/food chain

Undesirable substances of heavy metals (Cd, Pb, Hg, As, Cr) as well as essential heavy metals (Fe, I, Co, Cu, Mn, Mo, Se) have potential adverse effects on livestock directly, but can also enter the food chain through animal consumption and thus represent a risk for humans. Concerning the evaluation of exposure levels to toxic metals or to toxic doses, it is important to consider that the dietary intake can be influenced by several factors: management, type and quality of raw materials, additives, soil ingestion and accidental contamination. Episodes of acute toxicity are uncommon, with the exception of accidental exposure. Heavy metals are potentially dangerous due to their toxicity, bioaccumulation and bio-magnification when found within living tissues, and are stored more quickly than they are excreted. Heavy metals are considered contaminants or undesirable substances (As, Cd, F, Pb, Hg) when they are not intentionally added to food, but may reach the feed and food chains throughout different sources (Jarup, 2003).

The carry-over of orally administered metals into animal-origin food (milk, eggs, meat) (Table 7) is related to the rate of absorption, bioaccumulation, metabolism and excretion (Cheli et al., 2013). These biological phenomena largely vary depending on the chemical form, are dose- and/or time-dependent (especially for some bio accumulative elements such as cadmium), and are influenced by other factors such as the interaction with other compounds (e.g. Cd greatly interferes with essential elements such as Cu and Zn) (Fink-Gremmels, 2012).

Table 7. Effects of different essential and nonessential heavy metals as contaminants on human health, the specific carry-over effects and their sources.

Heavy metal contaminants	Kind of element	Effects associated with human health	Carry-over to animal tissues and products	Source	Reference
Chromium (Cr)	EHM[a]	Skin irritation and ulceration after acute exposure; kidney and liver malfunctions and circulatory and nervous system damage after long-term exposure.	No data.	Metal alloys and pigments and other materials.	Wuana and Okieimen (2011)
Cadmium (Cd)	NHM[b]	Kidney and liver damage, skin irritation, ulceration, enzyme inactivation, lung cancer.	Liver and kidney: dose and time dependent. Muscle: very low (independent of the level of dietary exposure). Milk and eggs: very low or absent (<0.05%).	Tobacco smoking, whole grain cereals, fruit, root vegetables and wild mushrooms, offal (kidney and liver) of mammals and shellfish (oysters and scallops).	Food Safety Authority of Ireland (2009); EFSA (2009)
Lead (Pb)	NHM	Impaired development, lower IQ, shortened attention span, hyperactivity, and mental deterioration in children. Loss of memory, nausea, insomnia, anorexia, weakness of the joints, injury to the brain, nervous system, red blood cells, and kidneys in adults.	Muscle low and not significant Milk 0.1-1%.	Leafy vegetables (lettuce), root crops (carrots), fish and shellfish offal (liver and kidney), cereals and tap water.	Wuana and Okieimen (2011); EFSA (2010)
Mercury (Hg)	NHM	Damage to the central nervous system (neurotoxicity) and the kidney.	Limited information and no dose-response studies available.	Fish and fishery products.	Food Safety Authority of Ireland (2009)
Arsenic (As)	NHM	Skin damage, increased risk of cancer, and problems with circulatory system.	No data.	Fish, seafood (crab) and animal offal.	

[a] EHM – essential heavy metal;
[b] NHM – nonessential heavy metal.

With regard to Cd, Pb, As and Hg different studies showed that the carry-over to milk, egg and muscle is generally low if animals are fed with a standard diet (with a concentration of heavy metals below the EU maximum permissible levels). Conversely, with a higher dietary toxic metal exposure, a general increase in residues in the specific accumulation organs (liver, kidney, bones) has been observed (Thirulogachandar et al., 2014). Thus, the control of the animal input could be an effective strategy to reduce human health risks related to the consumption of animal-origin products.

The accumulation of heavy metals varies significantly from one tissue to another within an animal, and between animals. Higher As concentrations have been detected in breast meat, the essential edible part of poultry; and kidney and gizzard showed the highest content of Cd and Cr, respectively (Mohammed et al., 2013). A positive correlation between dietary intake and concentration in broiler organs (muscles, liver, and skin) has also been demonstrated for essential metals as Fe and Mn (Rehman et al., 2012)

Among animal products, eggs are also a possible source of heavy metal contamination. Radu-Rusu et. al. (2013) compared the heavy metal transferability in improved cages and free range reared hens, and discovered that free-range eggs had higher concentrations of heavy metals, compared to the conventionally-produced eggs, due to the intense soil contamination with these pollutants. The Cd level was 0.018-ppm vs 0.023 ppm in the free-range group; 2.591 ppm vs. 2.734 ppm for essential Cu, and the essential Zn content was 5.386 ppm in improved cages vs 5.522 ppm.

Ruminants are less susceptible to As toxicosis and do not show any sign of toxicity unless the concentration is more than 200 to 300 mg of inorganic As/kg of feed (Kochare and Tamir, 2015). Dairy cows are more susceptible to the accumulation of Cd and Pb than beef cattle, however both suffer from mercury toxicity. This heavy metal, especially in the form of methyl, is highly toxic and can lead to incoordination, decline in awareness, alopecia, and visual and gastrointestinal disorders. García-Vaquero et al. (2011) investigated intensively farmed beef cattle and demonstrated that essential Cu accumulation in the liver had negative effects on animal performance, and found a reduced feed intake and average daily gain. This decreased growth performance may be due to the production of reactive oxygen species (ROS) following excess supplementation with Cu.

Fish containing the valuable proteins and omega-3 polyunsaturated fatty acids and could represent source of heavy metal exposure to humans (Nnaji et al., 2011). According to Qiu et al. (2011), heavy metal accumulation in fish tissues depends largely on their concentrations in water, in prey or commercial feed. Dangerous heavy metals of interest include Hg, As which

are and carcinogenic. However, heavy metals tend to accumulate more in the visceral tissues of fish (liver, kidney, intestines etc), which are normally discarded during the manufacturing processes, than in the muscles (Nnaji et al., 2011). Qui et al. (2011) found significant relationships between the concentrations of essential Cu and Zn and lipid contents in two farmed fish species (pompano and snapper). Their findings indicate that lipid content may be an important factor regulating the bioaccumulation of these metals. Cu is an essential metal for both fish and humans, however Cu poisoning induces gill, liver and kidney damage in fish, leading to mortalities.

3.6. Mineral nutrition in livestock

Livestock nutrition plays a pivotal role not only in guaranteeing the animal requirements and thus preventing nutritional deficiency, but also in improving animal health and welfare, productivity, food safety and also in controlling environmental pollution. All animals require mineral nutrition including some heavy metals that have been demonstrated to be essential nutrients. Minerals, such as Co, Cu, Fe, I, Mn, Mo, Se, and Zn, are part of the numerous enzymes that coordinate many biological processes, and consequently are essential to maintain animal health and productivity (Lopez-Alonso et al., 2012a). Essential metals perform four important types of function: structural, physiological, catalytic, and regulatory (Suttle, 2010).

Many essential trace elements used in intensive livestock are found in manure, in direct proportion to the quote supplied over the minimal requirements. From a mineral nutrition point of view, and in order to prevent mineral deficiencies that could compromise the production, commercial feeds are often supplemented with minerals in order to promote the optimum growth rate, functional bioactivity and antimicrobial properties. For example, Se is naturally present in many foods, such as yeast. It plays a critical role in reproduction, DNA synthesis, hormone metabolism, and protects the body from infection, oxidative damage and has an important bioactive role related to a decrease in the susceptibility to carcinogens (Dai et al., 2016).

Farmers usually balance animal diets with minerals according to the maximum acceptable levels established by EU authorities. Nevertheless, the maximum permitted amount in feed is usually greater than the minimum requirement, resulting in the wide diffusion of minerals into the environment. For example, the minimum nutrient requirement for Zn (NRC, 2014) ranges between 50 to 100ppm in different grow phases, however it is often used as an additive thus taking the level to 150ppm, which is the maximum acceptable level established by the European authorities.

Although, the net requirements of essential metals are lower than the dietary needs, different aspects need to be considered in order to establish the optimal concentration in feed: genetic influences, dietary factors, interaction among nutrients, bioavailability, and subclinical toxic effects. The optimal mineral supplementation, which is represented as a band between the adequate and inadequate toxic dietary concentration established by the dose/response, should also consider unnecessary levels excreted into the environment. The ban on antibiotic growth promoters (EU Reg. 1831/2003) in livestock led to the study of alternative compounds (Rossi et al., 2013; Rossi et al. 2014a), however it increased the use of some minerals as growth stimulants and to prevent enteric diseases in pigs.

High doses of Zn in the form of premix have thus been widely used in several EU countries. Zn is essential in the maintenance and restoration of barrier integrity, protection against pathogens and modulation of the immune system, promoting antibody production against intestinal pathogens (Rossi et al. 2014c). In addition, Zn may reduce diarrhoea and increase growth rates in weaning piglets (Sales, 2013 and Walk et al., 2015). Although the pharmacological use of Zn (2500–3000 mg of Zn/kg), available with a veterinary prescription, can reduce intestinal disorders after the weaning of pigs, from a nutritional and ecological point of view, a better strategy would be to identify and counter the main cause of diarrhoea (Rossi et al. 2014c).

Cu is another important mineral that is profoundly connected with livestock production. When this trace element is added to the diet of fattening pigs, it causes a faster growth and better feed-conversion ratio (Polen and Voia, 2015). In pigs, dietary concentrations of 150–250 mg of Cu/kg can maximize growth performance without exposing animals to any risk of poisoning. Also in poultry production Cu, Zn and Mn prevents some diseases: Cu prevents anemia, while Zn and Mn act as catalysts in many enzymatic and hormonal reactions (Suleiman et al., 2015). Although corn tissues cultivated in soil with high amounts of Cu do not accumulate it at toxic levels, Cu represents an environmental concern, and can enter into the human food chain by accidental soil ingestion, contamination of edible plants by soil, or by the consumption of contaminated products of an animal food origin (Alfthan et al., 2015).

In order to develop a sustainable nutritional strategy, the maximum permissible levels should not be considered as optimal, and mineral supplementation should be established in terms of the desirable limits, which should be lower than the legal ones (Eu et al., 2007).

In conventional farms, minerals can be supplemented in different forms. Inorganic salts, such as sulphates, carbonates, chlorides, and oxides, are the most common ones. When ingested, these salts are broken down in the digestive tract to form free ions which are then absorbed

(Lopez-Alonso et al., 2012b). In order to ensure the extranutritional effect of some elements on animals, the concentrations of these salts often exceed the physiological requirements, causing faecal excretion. Thus, there has been increasing interest in chelated compounds as hyperavailable mineral sources, thus reducing the dispersal of minerals in manure. Various studies have shown that chelated minerals can be included at much lower levels without compromising performance, minimizing nutrient excretion and the overall environmental impact (Lopez-Alonso et al., 2012b). However more light needs to be showed on this matter, in fact faecal Zn excretion is related to the dietary Zn concentration rather than the source.

For an optimal mineral supplementation, the various interactions among minerals and diet components need to be considered. Positive and negative nutrient interactions, involve the impact of the nutrient on another nutrient's bioavailability, including absorption and use. For example, Cu interacts mainly negatively with Mn, Zn and Fe (García-Vaquero et al., 2011).

Various proteins bind and carry certain minerals including Fe, Cu and Ca and thus an inadequate protein intake may impair the function of these nutrients (Collins et al., 2010). The quality and quantity of dietary fibre can negatively influence the absorption of several minerals, including Ca and Fe. In monogastric animals, phytic acid, found in cereals and legumes, can bind minerals to insoluble complexes, thus decreasing the absorption of 2+ chemical configurations. The interaction of vitamins and minerals has also been described in several metabolic situations and is still under investigation. Many trace elements are recognized as oxidants, which may deteriorate animal feed, in particular during long-term storage at high temperatures (Medardus et al., 2014).

A well-balanced diet is generally recommended in order to meet the requirements of all nutrients, considering possible interactions among nutrients, preventing deficiencies and chemical excesses or imbalances. For sustainable animal production and to develop effective approaches to preserving long-term soil and water quality strategies from heavy metal pollution, it is necessary to understand the nutritional basis of the interactions between organisms and the environment.

3.6.1. Heavy metals in feed: worldwide situation

Farming practices vary widely according to the global soil and climatic conditions, thus heavy metal contamination in feed can have divergent concentrations and strictly depends on the location and legal restrictions (Table 8).

Table 8. Comparison of content of heavy metals in feed in the USA, China, and England and Wales.

Source	Heavy metal	Kind of element	Concentration	Reference
Dairy feed	As	US[a]	11-33 mg/kg	Dai et al., 2016 – USA
			0,01-6,123 mg/kg	Zhang et al., 2012 – China
			<10 mg/kg	Wang et al., 2013 – China
			0,1-4,13 mg/kg	Nicholson et al., 1999 – EW[d]
	Cd	US	5 ppb to 82 ppb	Dai et al., 2016 – USA
			Nd[c] -23,25 mg/kg	Zhang et al., 2012 – China
			< 10 mg/kg	Wang et al., 2013 – China
			0,1-3,59 mg/kg	Nicholson et al., 1999 – EW
	Pb	US	12-349 mg/kg	Dai et al., 2016 – USA
			< 10 mg/kg	Wang et al., 2013 – China
			1,0-8,23 mg/kg	Nicholson et al., 1999 – EW
	Cu	EHM[b]	37,8 mg/kg	Dai et al., 2016 – USA
			2,73-114,68 mg/kg	Zhang et al., 2012 – China
			15,7 mg/kg	Wang et al., 2013 – China
			2-21,3 mg/kg	Nicholson et al., 1999 – EW
	Cr	EHM	4,91 mg/kg	Dai et al., 2016 – USA
			<10 mg/kg	Wang et al., 2013 – China
	Zn	EHM	11,07-346,12 mg/kg	Zhang et al., 2012 – China
			73,0 mg/kg	Wang et al., 2013 – China
			6-83 mg/kg	Nicholson et al., 1999 – EW
Pig feed	As	US	0,02-13,03 mg/kg	Zhang et al., 2012 – China
			< 10 mg/kg	Wang et al., 2013 – China
	Cd	US	Nd-31,65 mg/kg	Zhang et al., 2012 – China
			< 10 mg/kg	Wang et al., 2013 – China
	Cu	EHM	169,9 mg/kg	Dai et al., 2016 – USA
			2,3-1,137 mg/kg	Zhang et al., 2012 – China
			36,9 mg/kg	Wang et al., 2013 – China
			18-217 mg/kg	Nicholson et al., 1999 – EW
	Zn	EHM	37,37-598,32 mg/kg	Zhang et al., 2012 – China
			103,3 mg/kg	Wang et al., 2013 – China
			150-2920 mg/kg,	Nicholson et al., 1999 – EW
Poultry feed	Cu	EHM	132,7 mg/kg	Dai et al., 2016 – USA
			2,88-98,08 mg/kg	Zhang et al., 2012 – China
			17,0 mg/kg	Wang et al., 2013 – China
			24,8-52,4 mg/kg (broiler)	Nicholson et al., 1999 – EW
	Zn	EHM	52,62-150,97 mg/kg	Zhang et al., 2012 – China
			99,1 mg/kg	Wang et al., 2013 – China
			106-169 mg/kg	Nicholson et al., 1999 –EW

[a] US – undesirable substances in animal feed;
[b] EHM – essential heavy metal;
[c] Nd – Non-detectable;
[d] EW – England and Wales.

To be able to predict the risk of exposure to toxic doses of metals, it is important to consider the production system. Extensive ruminant farms, both beef cattle and dairy, need land to produce forage (hay, straw and silage) as energy sources for the ruminal symbiotic microflora (Zaninelli et al., 2015). Naturally, all soils contain different concentrations of heavy metals. Furthermore, such components are also added to agricultural soils through the application of mineral and organic fertilizers, direct defecation and urination by animals, and pesticides (Kochare and Tamir 2015). In these cases, exposure in animals may vary in relation to the quality of the soil, the use of inorganic fertilizers and anthropogenic activities. Animals in intensive systems usually receive concentrate feed, with raw materials from a global market supplemented by mineral additives. By accurate monitoring in intensive systems, the control of the input of heavy metals is easier than in extensive systems.

Feed additives are widely applied in animal production in all described locations and Cu and Zn are present in feeds largely. The content of heavy metals, especially Cu and Zn in swine feed has been found to be higher than in poultry feed or in cattle feed. According to Dai et al. (2016) more than half of the Wisconsin (USA) dairy farms used feed rations containing Cu above the recommended levels. Zhang et al. (2012) reported that in northeast China Cu, Zn and As were found in all feed samples. According to Wang et al. (2013) in China (Jiangsu Province), median concentrations of Zn were the highest heavy metals found, followed by Cu. The median concentrations of undesirable substances such as Hg, As, Pb, Cd, and Cr in all feeds were below 10 mg/kg (Table 8). This is probably related to the different management of raw materials and feed, animal species as well as different legal limits.

The concentration of toxic undesirable metals, such as Cd and Pb, was higher in forage than in concentrate feed materials and in particular in herbage cultivated near industrial areas. This is probably related to the contamination of forage with soil and not to the plant uptake. Instead, the main sources of As and Hg in feeds are represented by non-plant materials such as products of marine origin.

3.7. Heavy metals in livestock manure

Heavy metals can be introduced into agricultural soil through manure. The input of organic waste to agricultural soil increases organic matter, introduces nutrients, improves soil structure, and increases nutrient absorption by plants, which improves soil fertility and quality (Gul et al., 2015). Despite, the considerable fertilizer value of slurry, it may be abundant in Zn, Cu and other heavy metals derived from animal intake (Jakubus et al., 2013). The heavy metal

composition of composts varies widely depending on the geographical location, sources and composting process (Faridullah et al., 2014).

Ingelmo et al. (2012) reported on digested sewage sludge (collected from Spanish farms), during the compost process. They found that the heavy metal content varies and total Zn, Pb, Cu and Ni content increased during the composting process. On the final composting day, the content was ranked Zn>Pb>Cu>Ni>Cd (Table 9). Thus, the total content of heavy metals depends on the organic matter transformation, which may influence the bioavailability of metals and in the end render the metals in more available forms.

Table 9. Essential and nonessential heavy metal content ($mg \cdot kg^{-1}$) in sludge-based compost on different days of composting (Ingelmo et al., 2012).

Time (days)	0	14	84	140
Cu (EHM)	37,70	41,30	43,10	49,50
Ni (EHM)	2,24	2,38	2,69	2,76
Zn (EHM)	259,80	262,10	267,10	278,20
Pb (NHM)	45,30	49,50	53,70	57,40

EHM – essential heavy metal;
NHM – nonessential heavy metal.

Faridullah et al. (2014) have shown that acid-extracted metals were also higher in composted manure than fresh manure. The authors collected animal-composted waste samples (Abbottabad District, Pakistan). They were ranked Fe>Hg>Mn>Zn>Ni. In addition, Fe, Mn and Hg concentrations were higher in the composted manures, whereas Ni and Zn showed their maximum concentrations in fresh manures. The maximum Fe concentration was detected in composted buffalo manure. Faridullah et al. (2014) observed a different trend in metal extraction. The high content of Fe was related to the different feed composition or products of fish origin. According to Jakubus et al. (2013) tested slurries (collected from Dutch farms) containing Cu and Zn were from 1.5 to 3.0 times higher in swine slurry than cattle slurry. Nicholson et al. (2003) showed that in England and Wales, the highest metal concentrations for swine and cattle and poultry livestock manures were Zn and Cu. In sewage sludge the highest concentrations of heavy metals were also Zn, Cu and also Pb, Cr. In China, the highest concentrations for swine livestock manures were: Zn, Cu, Cr and Pb; and for cattle manures: Zn, Cu, Pb and Cr. The contents of trace elements in animal manures increased over a decade, with the use of feed additives (Luo et al., 2009). Zhang et al. (2012) showed that in north-east China, the contents of Cu and Zn in manures of different size farms (small, medium and large farms) were significantly higher than other metals detected. In cattle and chicken manure, there was no significant difference in the content of heavy metals from farms of different sizes (Table

10). In China, in the last decade an increase in the content of Zn in manure has been found. This is related to the high content of Zn in animal additives, which have usually resulted in a higher concentration in the manure.

Table 10. Concentration of different essential and nonessential heavy metals in selected livestock manures in different countries.

Area	Heavy metal			Kind of element	Source of heavy metals			
					Cattle slurry	Swine slurry	Poultry slurry	Buffalo Slurry
Netherlands (Jakubus et al., 2013)	Zn	mg·kg^{-1}		[f] EHM	73,7	186,2	-	-
	Cu				296,3	644,7	-	-
England and Wales (Nicholson et al., 2003)	Zn	mg/kg ds		EHM	170,0	650,0	217,0	-
	Cu				45,0	470,0	32,0	-
	Ni				6,0	14,0	4,0	-
	Cr				6,0	7,0	2,0	-
	Pb			[g] NHM	7,0	80,0	3,3	-
Pakistan (Faridullah et al., 2014)	Zn	mg·kg^{-1}	CM[a]	EHM	163,0	-	187,0	145,0
			FM[b]		150,0	-	160,0	170,0
	Mn		CM		430,9	-	466,1	475,3
			FM		437,0	-	438,0	456,7
	Fe		CM		1825,5	-	1873,0	2147,0
			FM		1664,0	-	1806,3	1821,2
	Ni		CM		71,7	-	66,2	83,9
			FM		74,2	-	74,0	81,5
	Hg		CM	NHM	771,4	-	787,0	788,3
			FM		783,7	-	758,5	728,3
China (Luo et al., 2009)	Zn	mg/kg d.w.		EHM	151,9	843,3	308,9	-
	Cu				46,5	472,6	102,0	-
	Cr				15,2	46,6	46,0	-
	Pb			NHM	15,7	10,1	20,6	-
China (Zhang et al., 2012)	Cu	mg/kg d.w.	S[c]	EHM	30,8	958,8	51,6	-
			M[d]		31,0	420,4	57,2	-
			L[e]		31,4	612,2	87,1	-
	Zn		S		119,1	674,7	268,2	-
			M		126,3	476,0	241,7	-
			L		136,1	691,6	384,2	-
	Cr		S		1,3	2,7	16,6	-
			M		1,1	4,2	224,8	-
			L		0,2	6,6	23,7	-
	Pb		S	NHM	1,9	2,9	2,2	-
			M		2,2	2,5	4,9	-
			L		2,7	2,4	4,4	-

[a] CM – composted manure,
[b] FM – fresh manure,
[c] S – small; animal population (head): cattle <100, chicken <2000, swine <200.
[d] M – middle; animal population (head): cattle 100-300, chicken >2000, swine 200-800.
[e] L – large; animal population (head): cattle >300, chicken >20000, swine >800.
[f] EHM – essential heavy metal;
[g] NHM – nonessential heavy metal.

The heavy metal contents of animal manures are largely a reflection of their content in the feed, which poses a high pollution risk to farmlands. Considering the different kinds of farms and different species, swine and poultry represent the most important sources of Zn and Cu pollution. This is also linked to the additives used in animal feed (Nicholson et al., 2003; Luo et al., 2009; Zhang et al., 2012, Jakubus et al., 2013). Consequently, swine and poultry farms may have the highest risk for agricultural lands. In terms of environmental protection, animal feed additives should be monitored based on the legal limits in each country (Zhang et al., 2012). Different approaches are also needed for reducing heavy metal inputs to agricultural land and to target policies for preserving long-term soil quality (Nicholson et al., 2003).

3.8. Heavy metal content in agricultural soil

The soil represents an important risk in terms of the livestock exposure to heavy metals due to accidental ingestion, contamination of forage, and absorption by edible plants. In most EU member states, but also in the rest of the world, complete inventories of soil are lacking. Quantifying the full extent of local soil pollution is therefore difficult, although this is an important further objective of the EU in the proposed Soil Framework Directive. According to the European Environment Agency (2006) measurements, there were a total of three million potentially contaminated locations in the EU, of which 250 thousand were actually contaminated (Dir. 2004/35/EC). Therefore, reducing heavy metal contamination in the soil is a strategic target for EU soil protection policies (Nicholson et al., 2003).

In various parts of the world widely different levels of trace elements in the agricultural industry have been observed. Zn pollution has become a general global problem. Zn contamination has reported in all described locations (Nicholson et al., 2003; Luo et al., 2009; Belon et al., 2012) (Table 11). In fact, Zn is monitored by authorities as it is responsible for eutrophication and water pollution. Depending on the country, there are main sources of Zn pollution: atmospheric deposition and livestock manure. The livestock industry contributes to Zn pollution as it is widely used in animal feed as additives. Authorities should thus monitor the pollution and create new strategies for the improved management of animal nutrition. This would help to prevent soil contamination and to build an approach based on ecological nutrition, which could remain sustainable development between economic development, social development and environmental protection. The pressure on agricultural land in China is almost nine times higher for Zn (187 742 $g/ha^{-1}/yr^{-1}$) and more than 14 times higher for Cu (71 824 $g/ha^{-1}/yr^{-1}$) compared to Germany (Zn – 21 237 $g/ha^{-1}/yr^{-1}$) and France (Cu – 4 869 $g/ha^{-1}/yr^{-1}$) which have the highest annual input of Zn and Cu in the total area of agricultural land, in all

described countries in Europe. These differences between China and Europe are probably related to different legal restrictions.

Table 11. Comparison of annual input of essential and nonessential heavy metals in soil for 1 million of ha (mln ha) yearly in various countries (adapted from Nicholson et al., 2003; Luo et al., 2009; Belon et al., 2012).

Total area of land (mln ha)		Cu g/ha^{-1}	Cr g/ha^{-1}	Ni g/ha^{-1}	Zn g/ha^{-1}	Cd g/ha^{-1}	Pb g/ha^{-1}
		EHM[g]				NHM[h]	
CH[a]	122	588,7	139,9	86,2	1 538,9	11,6	238,2
FR[b]	29	167,9	34,5	19,6	523,8	1,9	24,0
GR[c]	17	269,2	-	-	1 249,2	4,9	86,1
UK[d]	11.1	146,0	29,5	26,9	453,9	3,6	70,1
NL[e]	2	294,0	-	-	684,5	4,5	80,4
SW[f]	1.1	298,2	-	-	768,2	1,8	74,5

[a] CH – China;
[b] FR – France;
[c] GR – Germany;
[d] UK – United Kingdom;
[e] NL – Netherlands;
[f] SW – Switzerland;
[g] EHM – essential heavy metal;
[h] NHM – nonessential heavy metal.

In the EU in terms of the entire agricultural land area, atmospheric deposition has been reported to be the main source of most metals, ranging from 25% to 85% of total inputs (Nicholson et al., 2003). According to Luo et al. (2009), in China atmospheric deposition and livestock manures were also the predominant sources of trace elements in agricultural land. In agricultural soils in China, atmospheric deposition may be responsible for 43–85% of the total As, Cr, Hg, Ni and Pb inputs. The average atmospheric deposition flux of As in China is about 100 times higher than that in Europe. These sources are related to agricultural and industrial activities (Belon et al., 2012) (Table 12).

Table 12. Essential and nonessential heavy metals in atmospheric deposition rate yearly in the United Kingdom (Nicholson et al., 2003), France (Belon et al., 2012) and China (Luo et al., 2009).

Atmospheric deposition rate (mean)	Cu g/ha^{-1}	Cr g/ha^{-1}	Ni g/ha^{-1}	Zn g/ha^{-1}	As g/ha^{-1}	Pb g/ha^{-1}
	EHM				NHM	
UK[a]	57,0	7,5	16,0	221,0	3,1	54,0
FR[b]	8,0	2,4	0,5	55,8	0,5	7,7
CH[c]	108,0	61,0	58,0	647,0	28,0	202,0

[a] UK – United Kingdom;
[b] Fr – France;
[c] CH – China;
[d] EHM – essential heavy metal;
[e] NHM – nonessential heavy metal.

In England, livestock manure and sewage sludge are also important sources, responsible for an estimated 37–40% and 8–17% of total Zn and Cu inputs. According to Belon et al. (2012) in France animal manure, mineral fertilizers and pesticides are the predominant sources of heavy metals. Livestock manure was the predominant (>50%) source of Zn, Cu, Mo Ni, As, and Hg. Although the toxic metal concentration in feedstuff from unpolluted soil has been found to be in line with the safety limits established by the EU, a renewed inventory of metal inputs into agricultural soils is of immense importance in order to assess the environmental risks posed by contaminated agricultural soils (Luo et al., 2009).

3.9. Strategies to control heavy metal pollution

Controlling environmental losses and the spread of contaminants from livestock manure is essential in balanced production systems and in order to achieve the homeostasis of agriculture with natural habitats. Although, as in most European countries, spreading manure near to surface water and on frozen soil is illegal, the accidental release of farm waste to water has resulted in outbreaks of serious illnesses. There is thus a need for technologies and strategies to control these environmental problems. Efforts are also needed to close nutrient cycles on farms by recycling nutrients in livestock manure which will reduce pollution problems and limit heavy metals in soil (Petersen et al., 2007; Gerber et. al., 2014). Therefore, many studies have described strategies to control the content of heavy metals in livestock manure and in soil. Effective strategies should focus on a reduction in the heavy metal input/output ratio in livestock. Thus, different multidisciplinary approaches should be considered to reduce the animal intake, but also the excretion in faeces and the concentration in manure.

The manipulation of the diet could be a useful way to control the amount of manure produced together with its composition, because nutrients found in manure or in compounds derive from the fraction of the feed that is not absorbed by the animals. A formulated diet is needed that increases the efficiency of nutrient retention by animals, decreasing their excretion in faeces and urine and reduce the import of nutrients in feed and mineral mixtures from outside the farm (Petersen et al., 2007). For instance, in pigs and poultry, the use of industrial amino acids is a very efficient way to reduce nitrogen excretion. At the farm level this thus leads to a significant reduction in the import of protein rich feedstuffs, such as soybean meal. The inclusion of enzymes in the feed which improves the biological availability of some specific nutrients has been shown to be efficient in many species. Animal feeding plays an important role in the control of nutrient flows on livestock farms (Petersen et al., 2007). Using mineral supplements of trace minerals could help prevent the “waste”. The maximum permitted level

should not be considered as the ideal level for animal requirement and alternative innovative compounds to antibiotics but also to Zn and Cu should be used to control enteric diseases (Rossi et al., 2014b).

An excessive heavy metal output still can penetrate the soil and water from manures, thus there is a need for different technologies to remove the content of heavy metals from contaminated soil and water in agricultural land (He et al., 2005). To reduce the heavy metal output from livestock, different approaches to treat the manure have been studied and can be applied in the field.

Electroremediation which passes an electric current through liquid manure and metal ions are precipitated on an electrode, can reduce metal concentrations. However, at present the technology is unproven at the farm-scale and is unlikely to be cost-effective. From a whole-farm perspective, the recycling loop of manure back into food production should be as short as possible in order to minimize the environmental impact and ensure a high nutrient efficiency (Petersen et al., 2007).

According to Bhargava et al. (2012) phytoremediation is simple cleanup technology which has promising possibilities to eliminate metals from agricultural land, through the use of plants that accumulate large amounts of heavy metal contaminants. This technology was developed a few decades ago from the recognition that plants were capable of metabolizing toxic pesticides (Van Aken, 2009). It is perceived as an acceptable, cost-effective, and efficient, novel technology with acceptability among the communities. Phytoremediation comprises several techniques that use plants and associated microbes to remediate contaminated matrices, which are removed through transfer, containment, accumulation or dissipation. The fact that phytoremediation is usually carried out in situ contributes to its cost-effectiveness and may reduce the exposure of the polluted substrate to humans, wildlife, and the environment (Pilon-Smits, 2005). Depending on the conditions, the level of clean-up required, the plants used and the contaminants, phytoremediation can be divided into four types: phytoextraction, phytofiltration, phytostabilization and phytovolatilization (Thangavel and Subbhuraam, 2004; Ali et al., 2013).

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Phytoextraction is the uptake of contaminants from soil or water by plant roots and their translocation to and accumulation in above ground biomass (Rafati et al., 2011). Phytofiltration is the removal of pollutants from contaminated surface waters or wastewater (Mukhopadhyay and Maiti 2010). Removal reach by plants roots (rhizofiltration) or seedlings (blastrofiltration). Seeding roots or plants roots rose in aerated water absorb, precipitate and concentrate heavy metals (Thangavel and Subbhuraam 2004). Phytostabilization is used to reduce the mobility and bioavailability of pollutants in the environment, thus preventing their migration to groundwater or their entry into the food chain (Erakhrumen, 2007). Phytovolatilization is the uptake of pollutants from soil by plants, their conversion to volatile form and subsequent release into the atmosphere. Among these techniques phytoextraction is the main and most useful technique for removal of heavy metals and metalloids from polluted soils or water (Ali et al., 2013).

The effectiveness of phytoremediation is highly influenced by the bioavailability of metals in soil that depends on several factors: chemical composition, pH, geochemical characteristic of metals, environmental variables and agricultural soil management (Thangavel and Subbhuraam, 2004). Bioavailability can be increased by lowering pH of soil, using fertilizers, soil microorganisms, root exudates and adding chelating agents (Lone et al., 2008).

3.10. Conclusions

In the commercial agricultural industry, heavy metals are represented as both mineral nutrients and contaminants/undesirable substances. Although EU has established a comprehensive regulation to control their pollution, their spread at different level does not allow avoiding the presence of heavy metals in the food chain, and in the environment. The control of the animal input could be an effective strategy to reduce human health risks related to the consumption of animal-origin products and the environmental pollution by manure. The diets of livestock can be manipulated in order to reduce the quote of non-absorbed minerals and nutrients that can be present in the manure. To set up effective strategies against heavy metals the complex interrelationships in rural processes, the widely variability of farming practices, the soil and climatic conditions must be considered. Using the additives with more precision should be suggested in order to avoid spreading the contaminations to the environment. Innovative and sustainable approaches have discussed for the heavy metal nutrition ecology to control the environmental pollution from livestock-related activities.

3.11. References

Alfthan G, Eurola M, Ekholm P, Venäläinen ER, Root T, Korkalainen K, Hartikainen H, Salminen P, Hietaniemi V, Aspila P, Aro A 2015. Effects of nationwide addition of selenium to fertilizers on foods, and animal and human health in Finland: From deficiency to optimal selenium status of the population. Journal of Trace Elements in Medicine and Biology. 31, 142-147.

Ali H, Khan E, Anwar Sajad M 2013. Phytoremediation of heavy metals – concepts and applications. Chemosphere 91, 869–881.

Aragay G, Pons J, Merkoçi A 2011. Recent trends in macro-, micro-, and nanomaterial-based tools and strategies for heavy-metal detection. Chemical Reviews. 111 (5), 3433–3458.

Belon E, Boisson M, Deportes IZ, Eglin TK, Feix I, Bispo AO, Galsomies L, Leblond S, Guellier CR 2012. An inventory of trace elements inputs to French agricultural soils. Science of the Total Environment 439, 87–95.

Bhargava A, Carmona FF, Bhargava M, Srivastava S 2012. Approaches for enhanced phytoextraction of heavy metals. The Journal of Environmental Management 105, 103–120.

Cheli F, Pinotti L, Rossi L, Dell'Orto V 2013. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. Food Science and Technology 54(2), 307–314.

Collins J, Prohaska JR, Knutson MD 2010. Metabolic crossroads of iron and copper. Nutrition reviews: 68(3): 133-177.

Dai SY, Jones B, Lee K-M, Li W, Post L, Herrman TJ 2016. Heavy metal contamination of animal feed in Texas. Journal of Regulatory Science 01, 21–32.

Duruibe JO, Ogwuegbu MOC, Egwurugwu JN 2007. Heavy metal pollution and human biotoxic effects. International Journal of Physical Sciences. 2 (5), 112-118.

EFSA 2004b. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to lead as undesirable substance in animal feed. Adopted on 2 June 2004. EFSA Journal 71, 1-20.

Erakhrumen AA 2007. Phytoremediation: an environmentally sound technology for pollution prevention, control and remediation in developing countries. Educational Research and Review. Vol 2, 151–156.

Eu I, Yang XE, He ZL, Mahmood Q 2007. Assessing potential dietary toxicity of heavy metals in selected vegetables and food crops. Journal of Zhejiang University SCIENCE B: 8(1):1-13.

Faridullah F, Pervaiz A, Irshad M, Alam A, Mahmood Q, Ashraf M 2014. Trace elements characterization in fresh and composted livestock manures. The Journal of Hydrology 1(2), 1–6.

Fink-Gremmels J 2012. Animal Feed Contamination. Effects on livestock and food safety. A volume in Woodhead Publishing Series in Food Science, Technology and Nutrition.

Food Safety Authority of Ireland 2009. Mercury, lead, cadmium, tin and arsenic in food. Toxicology factsheet series, n°1, 1–13.

García-Vaquero M, Lopez-Alonso M, Benedito JL, Hernandez J, Gutiérrez B, Miranda M 2011. Influence of Cu supplementation on toxic and essential trace element status in intensive reared beef cattle. Food and Chemical Toxicology 49, 3358–3366.

Gerber PJ, Uwizeye UA, Schulte RPO, Opio CI, Boer de IJM 2014. Nutrient use efficiency: a valuable approach to benchmark the sustainability of nutrient use in global livestock production. Current Opinion in Environmental Sustainability Vol. 9–10, 122–130.

Giromini C, Rebucci R, Fusi E, Rossi L, Saccone F, Baldi A 2016. Cytotoxicity, apoptosis, DNA damage and methylation in mammary and kidney epithelial cell lines exposed to ochratoxin. Cell Biology and Toxicology. 32(3), 249–258.

Govind P, Madhuri S 2014. Heavy metals causing toxicity in animals and fishers. Research Journal of Animal, Veterinary and Fishery Sciences Vol. 2(2), 17–23.

Gul S, Naz A, Fareed I, Khan A, Irshad M 2015. Speciation of heavy metals during co-composting of livestock manure. Polish Journal of Chemical Technology 17 (3), 19–23.
Hambidge M 2003. Biomarkers of Trace Mineral Intake and Status. The Journal of Nutrition 133 no.3, 948–955.
He ZL, Yanga XE, Stoffell PJ 2005. Trace elements in agroecosystems and impacts on the environment. Journal of Trace Elements in Medicine and Biology. 19(2-3):125-40.
Ingelmo F, Molina MJ, Desamparados Soriano M, Gallardo A, Lapeña L 2012. Influence of organic matter transformations on the bioavailability of heavy metals in a sludge based compost. The Journal of Environmental Management 95, 104–109.
Jaishankar M, Tseten T, Anbalagan N, Mathew BB, Beeregowda KN 2014. Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary Toxicology. 7(2), 60–72.
Jakubus M, Dach J, Starmans D 2013. Biovailability of copper and zinc in pig and cattle slurries. Fresenius Environmental Bulletin Vol.22 no 4., 995–1002.
Jarup L 2003. Hazards of heavy metal contamination. British Medical Bulletin. 68, 167–82.
Kochare T, Tamir B 2015. Assessment of dairy feeds for heavy metals. The American Scientific Research Journal for Engineering, Technology, and Sciences. 11(1), 20–31.
Lone MI, He Z, Stoffella PJ, Yang X 2008. Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. Journal of Zhejiang University. 9, 210–220.
Lopez-Alonso M, Garcia-Vaquero M, Benedito, JL, Castillo C, Miranda M 2012a. Trace mineral status and toxic metal accumulation in extensive and intensive pigs in NW Spain. Livestock Science 146, 47–53.
Lopez-Alonso M 2012b. Trace minerals and livestock: not too much not too little. ISRN Veterinary Science. Volume 12, 1–18.
Luo L, Maa Y, Zhang S, Wei D, Zhu Y-G 2009. An inventory of trace element inputs to agricultural soils in China. The Journal of Environmental Management. 90, 2524–2530.
Maric M, Antonijevic M, Alagic S 2013. The investigation of the possibility for using some wild and cultivated plants as hyperaccumulators of heavy metals from contaminated soil. Environmental Science and Pollution Research. 20, 1181–1188.
Medardus JJ, Molla BZ, Nicol M, Morrow M, Rajala-Schultz P, Kazwala R, Gebreyes WA 2014. In-feed use of heavy metal micronutrients in U.S. swine production systems and its role in persistence of multidrug-resistant Salmonellae. Applied and Environmental Microbiology. 80(7), 2317–2325.
Mohammed AI, Kolo B, Geidam YA 2013. Heavy metals in selected tissues of adult chicken layers (Gallus spp). ARPN Journal of Science and Technology. 3(5), 518–522.
Mukhopadhyay S, Maiti SK 2010. Phytoremediation of metal enriched mine waste: a review. Global Journal of Environmental Research. 4, 135–150.
Nicholson FA, Smith SR, Allowayc BJ, Carlton-Smithd C, Chambersa BJ 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. Science of the Total Environment. 311, 205–219.
Nnaji JC, Uzairu A, Gimba C, Kagbu 2011. Heavy metal risks in integrated chicken-fish farming. Journal of Applied Sciences. 11: 2092-2099.
NRC 1998. Nutrient Requirements of Swine, 10th revised ed. National Academy Press, Washington, D.C.
NRC 2000. Nutrient Requirements of Beef Cattle, 7th revised ed. National Academy Press, Washington, D.C.
NRC, 2001. Requirements of Dairy Cattle, 7th revised ed. National Academy Press, Washington, D.C.
Petersen SO, Sommer SG, Béline F, Burton C, Dach J, Dourmad JY, Leip A, Misselbrook T, Nicholson F, Poulsen HD, Provolo G, Sørensen P, Vinnerås B, Weiske A, Bernal M-P,

Böhm R, Juhász C, Mihelic R 2007. Recycling of livestock manure in a whole-farm perspective. Livestock Science. 112, 180–191.
Pilon-Smits E 2005. Phytoremediation. Annual Review of Plant Biology. 56:15-39.
Polen T, Voia OS 2015. Copper effect of feed supplementation on growth performance in fattening pigs. Journal of Animal Science. 48(2), 28-30.
Qiu YW, Lin D, Liu JQ, Zeng EY 2011. Bioaccumulation of trace metals in farmed fish from South China and potential risk assessment. Ecotoxicology and Environmental Safety. 74, 284–293.
Radu-Rusu CG, Pop IM, Albu A, Bologa M, Radu-Rusu RM 2013. Transferability of certain heavy metals from hens to table eggs laid within different rearing systems. Lucrări Ştiinţifice-Seria Zootehnie. 59, 218–222.
Rafati M, Khorasani N, Moattar F, Shirvany A, Moraghebi F, Hosseinzadeh S 2011. Phytoremediation potential of Populus alba and Morus alba for cadmium, chromuim and nickel absorption from polluted soil. International Journal of Environmental Research. 5, 961–970.
Raubenheimer D, Simpson SJ, Mayntz D 2009. Nutrition, ecology and nutritional ecology: toward an integrated framework. Functional Ecology. 23, 4–16.
Rehman Ur K, Andleeb S, Mahmood A, Bukhari SM, Naeem MM, Yousaf K 2012. Assessment of heavy metals in different tissues of broilers and domestic layers. Global Veterinaria. 9(1), 32–37.
Rossi L, Dell'Orto V, Vagni S, Sala V, Reggi S, Baldi A 2014 (a). Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. Veterinary Research Communications. 38(1), 39–49.
Rossi L, Di Giancamillo A, Reggi S, Domeneghini C, Baldi A, Sala V, Dell'Orto V, Coddens A, Cox E, Fogher C 2013. Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. Journal of Veterinary Sciences. 14(3), 263–270.
Rossi L, Fusi E, Boglioni M, Giromini C, Rebucci R, Baldi A 2014 (b). Effect of Zinc oxide and Zinc chloride on human and swine intestinal epithelial cell lines. International Journal of Health, Animal Science and Food Safety. 2, 1–7.
Rossi L, Pinotti L, Agazzi A, Dell'Orto V, Baldi A 2014 (c). Plant bioreactors for the antigenic hook-associated flgK protein expression. Italian Journal of Animal Science. 13(1), 23–29 (b).
Sakakibara M, Ohmori Y, Ha NTH, Sano S, Sera K 2011. Phytoremediation of heavy metal contaminated water and sediment by Eleocharis acicularis. Clean: Soil, Air, Water. 39, 735–741.
Shabani N, Sayadi MH 2012. Evaluation of heavy metals accumulation by two emergent macrophytes from the polluted soil: an experimental study. Environmentalist. 32, 91–98.
Suleiman N, Ibitoye EB, Jimoh AA, Sani ZA 2015. Assessment of heavy metals in chicken feeds available in Sokoto, Nigeria. Sokoto Journal of Veterinary Sciences. 13(1), 17–21.
Suttle NF 2010. Mineral Nutrition of Livestock, 4th Edition. Cabi, Oxfordshire.
Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ 2012. Heavy metals toxicity and the environment. Experimental Neurology. 101, 133–164.
Thangavel P, Subbhuraam C 2004. Phytoextraction: role of hyperaccumulators in metal contaminated soils. Proceedings of the National Academy of Sciences. Part B 70, 109–130.
Theron AJ, Tintinger GR, Anderson R 2012. Harmful interactions of non-essential heavy metals with cells of the innate immune system. Clinical Toxicology. 1–10.
Thirulogachandar AME, Rajeswari M, Ramya S 2014. Assessment of heavy metals in Gallus and their impact on human. International Journal of Scientific and Research Publications. 4(6), 1–8.

Walk CL, Wilcock P, Magowan E 2015. Evaluation of the effects of pharmacological zinc oxide and phosphorus source on weaned piglet growth performance, plasma minerals and mineral digestibility Animal. 9(7), 1145–1152.

Wang H, Dong Y, Yang YS, Toor G, Zhang X 2013. Changes in heavy metal contents in animal feeds and manures in an intensive animal production region of China. Journal of Environmental Sciences. 25(12), 2435–2442.

Zaninelli M, Rossi L, Costa A, Tangorra FM, Agazzi A, Savoini G 2015. Monitoring of goats' health status by on-line analysis of milk electrical conductivity [Monitoraggio dello stato di salute delle capre attraverso l'analisi on-line della conducibilità elettrica del latte]. Large Animal Review. 21(2), 81–86.

Zhang F, Li Y, Yang M, Li W 2012. Content of heavy metals in animal feeds and manures from farms of different scales in northeast China. International Journal of Environmental Research and Public Health. 9, 2658–26.

Experiment 1:
Evaluation of concentration of heavy metals in animal rearing system

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4.1. Abstract

Animal manure is one of the diffusion routes of heavy metals and metalloids into the environment, where the soil can accumulate them. Heavy metals and metalloids can then be released into groundwater sources, be absorbed by crops, and enter the food chain with negative effects for human and animal health. The aim of this study was to evaluate the concentration of heavy metals and mineral nutrients from modern animal rearing systems in order to develop effective strategies to increase the sustainability. Samples of feed (n=24: n=16 from swine, n=8 from cattle), faeces (n=120: n=80 from swine, n=40 from cattle) and water (n=8), were collected from eight typical intensive swine and cattle farms located in northern Italy. All samples were analysed for the humidity and the principal components. The samples were also dried, mineralised and analysed by ICP-MS to detect the following elements: Na, Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, and Pb. The swine diets represented the highest amounts of Zn and Cu, with an average concentration for the finishing and weaning phases of Zn: 1737.9±301.3; 821.7±301.3; Cu: 133.8±11.6; 160.1±11.6 mg/kg as fed, respectively. The faecal content reflected the heavy metal composition from feed. The average content of cattle diets of Zn and Cu did not result higher than the maximum permitted levels. We observed that the swine manure represented the sources of Zn and Cu output into the environment. The Zn and Cu content should be monitored strictly in line with agroecology principles.

4.2. Introduction

Heavy metal and metalloid (HMM) pollution has become a serious problem in agriculture (Bhargava et al. 2012). The soil accumulates HMMs without apparent toxic effects; however HMMs can be released into water, be absorbed by crops, and enter the food chain in high concentrations, with negative effects for human and animal health (Jarup 2003). The major causes of HMM diffusion into the environment include the geological characteristics of soil, industrial and anthropic activities, atmospheric deposition, sewage sludge, animal manure, agrochemicals, and inorganic fertilizers (Nicholson et al. 2003). The strategy for the minimization of HMM effects is to reduce the environmental and human exposure to these elements.

Animal production thus represents a possible source of HMMs and also a key link in the food chain. The major HMMs input in pig livestock is represented by feed, which should be controlled in order to prevent the excessive spread of HMMs into the environment (Adewole et. al. 2016). Thus, HMMs should be monitored strictly in line with the modern principles of agroecology and in order to increase sustainability (Dumont et al. 2013).

HMMs can enter animals' diet as both contaminants or undesirable substances, and also as essential nutrients (Fink-Gremmels 2012; Hejna et al. 2018). For contaminants such as cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As), the maximum levels in certain foods have been established by regulation (2002/32/EC). In cases where the threshold is exceeded, they are not permitted in food and feedstuff. On the other hand, minerals such as cobalt (Co), copper (Cu), iron (Fe), iodine (I), manganese (Mn), molybdenum (Mo), selenium (Se) and zinc (Zn) are part of the numerous enzymes that coordinate many biological processes, and consequently should be used to supplement animals' diet in accordance with the authorized levels (EC N° 1831/2003; Lopez-Alonso et al. 2012).

The bioavailability of these minerals, that can influence their presence in the manure ranging from 10% to 80%, is influenced by factors such as animal status (age, production, health and physiological status), the chemical composition of the mineral source (organic vs. inorganic), and the diet composition. Nutrients that are not retained by the animal or exported in livestock products are excreted through manure, composed of animal faeces and urine (García-Vaquero et al. 2011; Fink-Gremmels, 2012; Bastianelli et al. 2015).

The manure is frequently used as a valuable organic fertilizer containing a broad range of nutrients such as nitrogen, phosphorus, potassium, as well as micronutrients (Nicholson et al. 2003; Moral et al. 2005, Chardon et al. 2012, Jakubus et al. 2013). HMMs output from intensive animal production varies considerably in relation to countries and farming system (Hejna et al.

2018). According to Nicholson et al. (2003) in England and Wales the Zn and Cu output from swine was higher in swine manure compared to cattle and poultry manure (650.00, 470.00 mg/kg DM, respectively). Moreover, Hölzel et al. (2012) reported the concentration of Zn in swine slurry ranged from 93.00 to 8239.00 mg/kg DM in Germany (Bavaria). Shi et al. (2019) reported higher average concentration of Zn and Cu in swine manure compared to cattle and sheep manure in China (1313.00, 514.70 mg/kg, respectively). Through the manure, a large number of metallic ions can be absorbed into the soil, thus interfering with the quality of cultivation or increasing soil pollution (Lopez-Alonso et al. 2012; Jakubus et al. 2013; Rossi et al. 2013, Rossi, Dell'Orto et al. 2014a; Gul et al. 2015; Liu et al. 2018).

Recently the EU has banned the inclusion of pharmacological levels of ZnO (EMA/394961/2017) in animal feed after 2022 because overall benefit-risk balance for additives containing ZnO remains negative. Due to recent legislation and lacking of the data about the HMMs pollution in Lombardy region, it is important to determine the situation in the field to monitor the Italian situation in the major part of intensive animal production. Thus, the aim of this study was to evaluate the concentration of heavy metals and mineral nutrients from feed, water and faeces in modern swine and cattle rearing systems in northern Italy in order to develop effective strategies to increase the sustainability.

4.3. Material and methods

4.3.1. Farm selection and sample collection

In this study, both pig and cattle randomly selected farms were considered. A total of four typical intensive swine farms (F1, F2, F3, F4) and four typical dairy cattle farms (F5, F6, F7, F8) located in northern Italy were included. All swine farms (F1-F4) leading to the Consortium of Parma. The breeding management including the organisation of safeguard, economic policy and quality control was developing according to Consortium regulations. Commercial swine farms selected to the survey consisted of a closed system with a number of sows ranging from 200 to 600, and farrowing, weaning and finishing phases. In each swine farm, dry feed, maize- and soybean-based meal, were supplied *ad libitum* in the growing phases and was formulated according to specific nutritional requirements for the production phase (NRC, 2012). Pregnant sows were fed complete dry feed (from 2 to 2.5 kg/head/day). Lactating sows on each farm, were fed *ad libitum* in order to obtain the maximum attainable consumption of nutrients. Water was supplied through nipple drinkers.

All indicated cattle farms (F5-F8) leading to the Grana Padano Consortium. The Italian Friesian dairy cattle farms enrolled in this study consisted of modern freestall barns, with

animals housed on padded mattresses or different types of bedding materials (sawdust or shavings, straw). These cattle farms consisted a number of lactating cows ranging from 200 to 600, and calves, and heifer's phases. The cows were fed a homogenous total mixed ratio (TMR) in feed lanes. The feed was composed of dry forage mixed with milled raw materials, which provide adequate nutrient intake for the milk production to meet the needs of dairy cows. Calves were fed commercial feed (dry forage and commercial milk replacement diet) individually. Water was available *ad libitum* through automatic waterers.

For each farm, the animal welfare, housing conditions, and health status were registered and assessed (Directive 98/58/EC, 2008/120/EC; Grandin, 2017).

All samples of feed and faeces were collected in the 10 days sampling procedure at the same hour in order to reduce the variability among periods. The feed samples were collected in order to guarantee the representativeness of samples according to the AOAC procedure (965.16). A total of 16 feed samples (500g each) from F1 (n=4), F2 (n=4), F3 (n=4) and F4 (n=4) were collected from different phases of production on swine farms (gestation, n=4; farrowing, n=4; weaning, n=4; finishing, n=4. Two different sampling procedures were adopted depending on the feed storage system used on the considered farms. The sampling from feed storage were adopted in order to reduce the variability between different feed distributions in the selected farms. In particular, where silos were used, ten subsamples were collected from at least 10 regions of silos which were then bulked together and thoroughly mixed to obtain a composite sample of approximately 500 g. Where the feed was kept in 25 kg bags, the commercial diets were sampled by taking grab subsamples from at least 10 regions of bags (50 g) within three-fourths of the depth. Individual grab subsamples were bulked and thoroughly mixed, and a composite sample of approximately 500 g was created.

A total of eight feed samples (500g each) from F5 (n=2), F6 (n=2), F7 (n=2) and F8 (n=2) were collected from different phases of cattle farms (calves, n=4; lactation, n=4). The commercial diet of calves and the TMR of lactating cows were sampled during the feeding period. At sampling, at least 10 subsamples were collected, taking care to prevent any spilling. Individual grab subsamples were bulked together and mixed thoroughly before a composite sample of approximately 500 g. All collected feed samples were placed in an airtight nylon bag, devoid of air, sealed tightly, and identified by a serial number.

A total of 80 faeces samples (50 g/each) from F1 (n=20), F2 (n=20), F3 (n=20) and F4 (n=20) were collected from different phases of swine farms (first week of gestation, n=20; farrowing, n=20; weaning, n=20; finishing, n=20). The fresh faecal samples from the first week after insemination and from lactating sows were collected individually from inside the cages.

The fresh faecal samples of weaning and finishing pigs were collected from five different areas of the pen floors.

A total of 40 faecal samples (50 g/each) from F5 (n=10), F6 (n=10), F7 (n=10) and F8 (n=10) were collected from different phases of cattle farms (calves, n=20; lactation, n=20). The fresh faecal samples from calves were collected individually from the cages. The fresh faecal samples from lactating dairy cattle were collected from different parts of fresh pats on the pen floor. Each sample was placed into a 50 ml polyethylene sterile tube, labelled and sealed immediately in order to avoid contamination. All the faecal samples were kept separately until the lab analysis.

Eight water samples from each selected farm were collected into 50 ml polyethylene sterile tubes, labelled and sealed immediately. All collected feed and faeces samples were transported in controlled conditions (4°C). Feed samples were then analysed immediately, and faecal samples were frozen and stored at -20°C for further analysis.

4.3.2. Chemical composition of feed and faecal samples

Each of feed samples (from F1 n=4, from F2 n=4, from F3 n=4, from F4 n=4; from F5 n=2, from F6 n=2, from F7 n=2, from F8 n=2) were mixed thoroughly and analysed for humidity as well for principal components (10g/DM each), such as crude protein (CP), crude fibre (CF), ether extract (EE) and ashes according to the Association of Official Analytical Chemists (AOAC, 2005; CR no. 152/2009). Dry matter (DM) was obtained by inserting mixed feed samples in preweighed aluminium bags and dried in a forced-air oven at 105°C for 24 h (AOAC 2005 method; proc. 930.15; CR No. 152/2009). All dried feed samples were then ground with a laboratory mill (Cyclone Sample Mill, Model 3010-019, pbi International, Milan, Italy). CP was measured according to the Kjeldahl method (AOAC 2005 method, proc. 2001.11). CF was determined by the Filter Bag technique (AOCS 2005 method, proc. Ba 6a-05). EE was determined by the Soxhlet method, with prior hydrolysis (European Commission Regulation No. 152/2009). Ashes were measured using a muffle furnace at 550°C (AOAC 2005 method; proc. 942.05).

Individual swine faecal samples (n=5 per each phase, each farm) collected from the same farm (F1, F2, F3, F4) and phase (gestation; farrowing; weaning; finishing) were thawed and combined to create one mega sample per each phase (from F1 n=4; from F2 n=4; from F3 n=4; from F4 n=4). Individual cattle faecal samples (n=5 per each phase, each farm) collected from the same farm (F5, F6, F7, F8) and phase (calves; lactation) were thawed and combined to create one mega sample per each phase (from F5 n=2; from F6 n=2; from F7 n=2; from F8

n=2). The faecal samples were analysed for the evaluation of DM, CP, CF, EE and ashes following the procedure described above.

4.3.3. Evaluation of minerals in feed, faecal and water samples by ICP-MS

Dried feed and faecal samples (0.3 g/DM each) were mineralized by the ultrawave single reaction chamber Microwave Digestion System (MULTIWAVE 3000; Anton Paar GmbH, Graz, Austria) in Teflon tubes filled with 10 ml of HNO_3 (65% concentrated) by applying a one-step temperature ramp (at 120°C in 10' and maintained for 10). The mineralized samples were cooled for 20 min and the homogenous sample solutions were transferred into the polypropylene test tubes. Feed samples (250 µl) were then diluted 1:40 with a standard solution containing an internal standard (100 µL) and H_2O (9.75 mL); while faecal samples (100 µL) were diluted 1:100 with standard solution containing an internal standard (100 µL) and HNO_3 (0.3 M, 10 mL).

An aliquot of 2 mgL^{-1} of an internal standard solution (^{72}Ge, ^{89}Y, ^{159}Tb) was added to the samples and calibration curve to obtain a final concentration of 20 μgL^{-1}. All samples were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Bruker Aurora M90 ICP-MS, Bremen, Germany) in order to detect the following elements: Na, Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, and Pb. The accuracy and precision of the results obtained using ICP-MS were evaluated using internal reference materials supplied by LGC Standards Company: sewage sludge (LGC 61812); poultry feed (LGC7173); and waste water (SPS-WW2 1). The typical polyatomical analysis interferences were removed using the Collision-Reaction-Interface (CRI) with an H_2 flow of 75mL/min through a skimmer cone.

4.3.4. Statistical analysis

In order to evaluate any statistically significant differences among the means all data were analysed using Proc Glimmix of SAS software (9.4. SAS. Inst. Inc., Cary, NC). The analysis accounted for the fixed effects of phases. Means were considered different when $P \leq 0.05$ and tended to differ if $0.05 < P \leq 0.10$. Tukey-Kramer studentized adjustments were used to separate treatment means within phases.

4.4. Results and Discussion

4.4.1. Chemical composition of feed and faecal samples

The collected diets showed a nutrient composition in line with the specific animal nutritional requirements (NRC 2001, 2012) for both swine and cattle feed within different phases. Nutritional requirements are different according to the animal's physiological stages

which were considered during the formulations of the rations (Jha and Berrocoso 2015; Patience et al. 2015; Wang et al. 2018).

The humidity content of the swine diets ranged from 8.83±1.60 for weaning, to 11.13±1.66% as f.w. for the farrowing phase (Table 13). Swine are monogastric and are usually reared on conventional high energy and protein rich rations (from 14.89±0.45 for gestation to 15.78±0.40 g/100g DM for the weaning phase) in order to guarantee the productivity. Although economic and environmental factors have compelled nutritionists to develop low-protein diets, proteins are the most important component in the swine diet (Miller, 2004; NRC 2012). Traditionally swine diets are formulated on the basis of crude proteins, which refer to the nitrogen content of the feedstuff per 6.25. The CPs of the analysed diet ensured the essential nutritional requirements for growth and gain efficiency.

As well as in swine diet, the calves need a high protein content in their diet. Thus, calves received dried feed with a higher level of crude proteins of milk origin compared to cows (20.35±0.18 vs. 13.86±0.82 g/100g DM, respectively; NRC 2001). On the other hand, the cow diet, represented by a total mixed ratio (TMR), showed a higher percentage of humidity (49.69±3.38 vs. 11.42±1.18% as f.w., respectively; Leonardi et al. 2005; Eastridge, 2006) and fibre (15.39±0.60 vs. 6.16±0.20 g/100g DM, respectively; Table 13). Fibre is an important nutrient for maintaining the functional parameters of the rumen and thus for sustaining animal health (Terré et al. 2013).

Table 13. The average chemical composition (on DM basis, excluded humidity) of feed from swine farms (F1 n=4, F2 n=4, F3 n=4, F4 n=4) per phase: gestation, farrowing, weaning and finishing and cattle farms (F5 n=2, F6 n=2, F7 n=2, F8 n=2) per phase: calves and lactation.

Composition	Swine				Cattle	
	Gestation	Farrowing	Weaning	Finishing	Calves	Lactation
Humidity (% as f.w.)	11.02±1.70[1]	11.13±1.66	8.83±1.60	9.81±1.28	11.42±1.18	49.69±3.38
Crude protein (%)	14.89±0.45	15.34±0.41	15.78±0.40	15.16±0.15	20.35±0.18	13.86±0.82
Nitrogen (%)	2.38±0.07	2.45±0.07	2.52±0.06	2.42±0.02	6.16±0.03	2.22±0.13
Crude fibre (%)	5.70±0.50	5.63±0.38	4.20±0.37	4.24±0.12	6.16±0.20	15.39±0.60
Lipids (%)	4.33±0.74	5.11±0.16	5.26±0.31	5.13±0.35	3.35±0.69	3.23±0.27
Ash (%)	7.81±0.50	7.88±0.62	6.57±0.42	6.50±0.42	7.95±1.15	7.02±0.31

[1] Data are presented as means and standard error of the means (SE).

Faeces represent the final product of the digestive process and mineral bioavailability of the nutrients from the diets (Bastianelli et al. 2015). Increasing humidity content was observed

in the swine faeces. The humidity content ranged from 70.13±2.46 for weaning to 73.52±1.10% as f.w. for the finishing phase. The faecal samples contained a high amount of water. Faeces resulting from the digestive process thus contained a higher amount of water (Le Goff and Noblet, 2001; Van Vliet et al. 2007). The protein content (CP) of swine faeces ranged from 11.51±0.74 for farrowing to 19.48±1.53 g/100g DM for the weaning phase. The high percentage of crude fibre in swine faeces is related to the monogastric physiology, which is not able to digest cellulose. The CF content (on DM basis) of faeces was three or four times higher than in the feed (5.63±0.38 vs. 18.31±1.36 g/100g DM for the farrowing phase; Table 14). Moreover, the ash content (on a DM basis) increased significantly compared to the swine feed (6.57±0.42 vs. 15.95±1.60 g/100g for weaning). This was related to the low absorption of minerals from the gut (Adewole et. al. 2016).

Table 14. The average chemical composition (on DM basis, excluded humidity) of faeces from swine farms (F1 n=4, F2 n=4, F3 n=4, F4 n=4) per phase: gestation, farrowing, weaning and finishing and cattle farms (F5 n=2, F6 n=2, F7 n=2, F8 n=2) per phase: calves and lactation.

Composition	Swine				Cattle	
	Gestation	Farrowing	Weaning	Finishing	Calves	Lactation
Humidity (% as f.w.)	72.78±1.75[1]	71.65±1.73	70.13±2.46	73.52±1.10	80.03±3.12	85.80±0.25
Crude protein (%)	12.98±1.04	11.51±0.74	19.48±1.53	19.02±1.56	20.61±3.57	14.15±0.74
Nitrogen (%)	2.08±0.17	1.84±0.12	3.12±0.24	3.05±0.25	3.30±0.57	2.26±0.12
Crude fibre (%)	17.39±1.45	18.31±1.36	15.28±1.69	14.96±0.76	18.89±2.21	25.20±0.99
Lipids (%)	4.02±0.89	3.88±0.73	6.86±0.72	8.23±1.26	4.00±1.34	1.87±0.29
Ash (%)	18.64±2.68	17.96±1.01	15.95±1.60	14.12±0.61	12.90±0.59	12.26±0.55

[1] Data are presented as means and standard error of the means (SE).

The high percentage of crude fibre in cattle faeces is related to the ruminant physiology (6.16±0.20 vs. 18.89±2.21 g/100g for the calf phase). The amount of fibre in cattle faces is a result of incomplete digestion or indigestible components (Bargo et al. 2002; Indugu et al. 2017; Table 14). In addition, the chemical composition resulting after the digestion process of the ruminants has been shown to have a higher percentage of ash compared to cattle feed. This is related, as in the case of swine farms, to a low nutrient bioavailability (Warly et al. 2017). The amount of water consumed by the livestock is influenced by the animal species, growth stage, feed intake, environmental temperature and the choice of feeding equipment. The method used to cool animals, wash barns can also increase the input of water in the manure.

4.4.2. Evaluation of HMs and minerals in feed, faecal and water samples by ICP-MS

Minerals are an essential part of the diet in sustainable animal production for the fulfilment of nutrition requirements and to maintain the appropriate animal growth. However, animal diet may also contain contaminants such as cadmium, lead, mercury and arsenic.

Our study showed that undesirable elements represented by arsenic (As), cadmium (Cd), lead (Pb), cobalt (Co), nickel (Ni) and molybdenum (Mo) did not exceed the threshold levels for feed established by regulation (2002/32/EC) for all the selected feed samples of swine and cattle farms. Thus, feed samples did not represent any apparent risk for the intensive swine and cattle production systems (Supplementary Table 1). These results are in line with other studies (Mendoza-Huaitalla et. al. 2010; Zhang et al. 2012; Wang et al. 2013; Adamse et al. 2017).

Supplementary Table 1. The average concentration of remain elements (on fed basis) in feed from swine farms (F1, F2, F3, F4) per phase: gestation, farrowing, weaning and finishing and cattle farms (F5, F6, F7, F8) per phase: calves and lactation.

Phases	Na	Mg	K	Ca	Cr	Mn	Fe	Co	Ni	As	Se
	% mg/kg as fed				mg/kg as fed						
Swine farms											
Gestation	0.26± 0.06	0.32± 0.04	-	0.55± 0.11	2.57± 1.01	149.92± 18.13	342.57± 46.73	0.23± 0.04	1.82± 0.26	0.14± 0.04	0.38± 0.13
Farrowing	0.25± 0.07	0.28± 0.01	-	0.43± 0.06	1.81± 0.44	137.51± 13.67	304.69± 36.49	0.22± 0.03	1.94± 0.23	0.33± 0.10	0.47± 0.14
Weaning	0.37± 0.04	0.20± 0.02	0.77± 0.05	0.39± 0.01	2.77± 0.32	133.24± 20.94	365.50± 36.68	0.26± 0.04	1.73± 0.19	0.37± 0.12	0.61± 0.21
Finishing	0.39± 0.05	0.21± 0.01	0.76± 0.00	0.39± 0.01	2.79± 0.32	132.26± 18.91	378.82± 30.04	0.26± 0.04	1.83± 0.24	0.61± 0.16	0.65± 0.20
Cattle farms											
Calves	0.62± 0.01	0.30± 0.01	0.85± 0.02	1.11± 0.29	0.83± 0.04	89.28±1 4.00	182.20± 17.59	0.18± 0.01	2.01± 0.07	0.09± 0.02	0.09± 0.00
Lactation	0.30± 0.07	0.17± 0.03	-	0.38± 0.05	0.45± 0.10	50.46±1 3.90	135.96± 20.17	0.24± 0.07	1.30± 0.39	0.07± 0.01	0.07± 0.04

Data are presented as mean and SE.

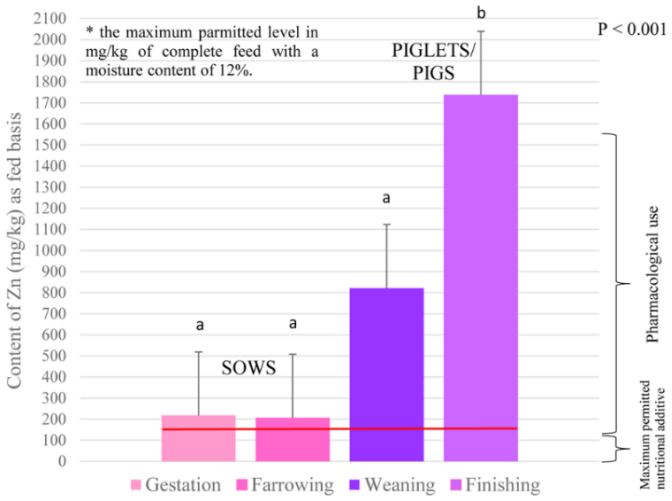
Iron (Fe) and manganese (Mn) did not exceed the threshold limits for feed in swine and cattle feed (EC N° 1831/2003). The average content of selenium (Se) in swine diets slightly exceeded the maximum permitted level (EC N° 1831/2003). This is probably not related to the mineral supplementation, but is a natural variation of the nutrients in raw materials (Gaudré and Quiniou, 2009; NRC 2012). Inversely, Se did not represent a risk for cattle feed and did not exceed the thresholds (EC N° 1831/2003). On the other hand, the contents of sodium (Na), magnesium (Mg), potassium (K), calcium (Ca) and chromium (Cr) met the range of nutritional requirements for both swine and cattle species (NRC 2001, 2012).

4.4.3. Content of Zn and Cu in swine and cattle farm feed samples

A different situation was found in relation to zinc (Zn) and copper (Cu) (Figure 9, Figure 10). The diets considered showed high levels of zinc, in particular for both the weaning and

finishing phases (821.7±301.3; 1737.9±301.3 mg/kg as fed, respectively). In our results, the wide range of dosage was similar to the feed from weaning phase in England (from 212.00 to 2350.00 mg/kg DM: Nicholson et al. (1999). Moreover, Mendoza-Huaitalla et al. (2010) reported in China the highest Zn average value in weaning phase from swine feed samples (1497.14 mg/kg, respectively). In contrary, Zhang et al. (2012) observed the Zn concentration in swine feed in China from 37.37 to 598.32 mg/kg. The estimated contents of Zn were significantly higher than 150 ppm, corresponding to the maximum permitted level as nutritional additives (EC, Reg. 1831/2003; EU 2016/1095). This is probably related to the pharmacological use of Zn, after veterinary prescription, to control enteric disorders which often appear during the growing phase. In fact, Zn is usually used to guarantee livestock productivity by controlling enteric pathogen bacterial infection as well as to enhance the integrity of the immune system (Luo et al. 2009; Hu et al. 2012; Liu et al. 2018). It is also crucial in these phases to reduce post-weaning diarrhoea and enhance animal growth performance related to the role of Zn in intestinal integrity (Zhang and Guo, 2009; Pearce et al. 2015).

Figure 9. The average concentration of zinc content in feed from different swine phases (gestation, weaning, farrowing, finishing) in considered swine farms (F1-F4) located in northern Italy. The red line represents maximum permitted level of zinc as nutritional additive in feed for pigs (150 mg/kg; EC N° 1831/2003; EU 2016/1095.).

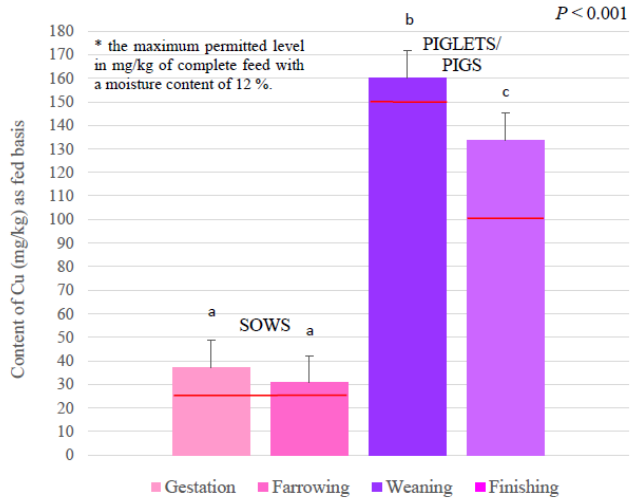


The average humidity content (% as f.w.) in swine feed (with SE): 11.02±1.70 for gestation; 11.13±1.66 for farrowing; 8.83±1.60 for weaning; 9.81±1.28 for finishing.
Data are presented as least squares means and SEM.

After the antibiotics ban in 2006 in Europe (EC, Reg. 1831/2003), there has been an increased use of high dosages of zinc oxide (ZnO). However, excessive exposure with higher concentrations of Zn has been linked to an increase of antimicrobial resistance, change in microbiota, accumulation of ions in vital organs, and environmental issues (Lyubenova et al.

2011; Dumont et al. 2013; Fusi et al. 2014b). The EU has thus banned the inclusion of pharmacological levels of ZnO after 2022 (EMA/394961/2017). In line with the major topics of agroecology, considering the needs of animals (health, welfare and nutrition productivity) and farmers (profitability and productivity) together with the environment, alternative solutions are required to control enteric disorders and implement the sustainability of pig livestock.

Figure 10. The average concentration of copper content in feed from different swine phases (gestation, weaning, farrowing, finishing) in considered swine farms (F1-F4) located in northern Italy. The red line represents maximum permitted level of copper as nutritional additive in feed for pigs (under 12 weeks – 170 mg/kg; above 12 weeks – 25 mg/kg; EC N° 2018/1039).



The average humidity content (% as f.w.) in swine feed (with SE): 11.02±1.70 for gestation; 11.13±1.66 for farrowing; 8.83±1.60 for weaning; 9.81±1.28 for finishing.
Data are presented as least squares means and SEM.

In pig diets, the amount of Cu in all phases resulted higher than maximum permitted level although at lower concentrations compared to Zn (Figure 10; weaning: 160.09±11.55; finishing: 133.75±11.55 mg/kg as fed, respectively). The results were in line with Nicholson et al. (1999), which reported in England the Cu concentration in feed for the weaning phase from 121 to 190 mg/kg DM. Moreover, Dai et al. (2016), in Wisconsin (USA) reported the similar average Cu concentration in pig feed (169.90 mg/kg). In contrary, in China, according to Wang et al. (2013) the average Cu concentration in pig feed was lower (36.90 mg/kg). Furthermore, compared to our results, Mendoza-Huaitalla et al. (2010) reported higher mean values of Cu in the weaning and finishing feed (240.29 mg/kg; 192.71 mg/kg, respectively).

In the sows, Cu was supplemented in the diet in order to maintain the fertility and improve reproductive performance. The most critical inclusion was observed in the finishing phase, where the average value was 30% higher than the maximum permitted level. The inclusion of Cu in young animals' rations is common practice to improve growth performance. It is related

to the bacteriostatic and bacterial properties of Cu, which may reduce the bacterial population in the intestine and protect against oxidative stress (Luo et al. 2009; Liu et al. 2018). Excessive exposure to high concentrations of Cu has been linked to the increase of the antimicrobial resistance and influence on the environment (Dumont et al. 2013). The EU thus recently decided to reduce (from 170 mg/kg to 100 mg/kg for up to four weeks after weaning) the maximum level of Cu in animal feed (EU Regulation, 2018/1039). When the ration is supplemented with minerals, it is important to consider any elements that may be contained naturally in the diet that could influence the total amount of the elements found in the manure.

Analysed water samples for Zn elements from swine farms for F1 were: 0.0001 mg/L; F3: 0.0553 mg/L; F4: 0.2540 mg/L and for Cu F1: 0.0029 mg/L; F3: 0.0748 mg/L and F4: 0.2117 mg/L. These results did not exceed the legal thresholds for drinking water (for Zn: 3 mg/L according D. Lgs. 152/2006 and for Cu: 2 mg/L according 1998/83/EC).

In order to increase the sustainability of livestock some aspects should be recognised. Bioavailability should be considered because the absorption of minerals from the gut is not complete and animals excrete from 70% to 95% of Cu and Zn. The mineral absorption is mostly influenced by the chemical form of salts used as additives in the diet (Suttle, 2010). In order to increase mineral absorption, and decrease the excretion, other sources of mineral additives should be considered (Liu et al. 2018). In addition, nutrient absorption is influenced by factors such as the interaction with other compounds in the diet (García-Vaquero et al. 2011; Fink-Gremmels, 2012, Hejna et al. 2018). Mineral-binding factors, naturally present in cereals and grains, such as the salt form of phytic acid (phytates) may also limit the absorption of minerals (Jondreville et al. 2007; Bohn et al. 2008).

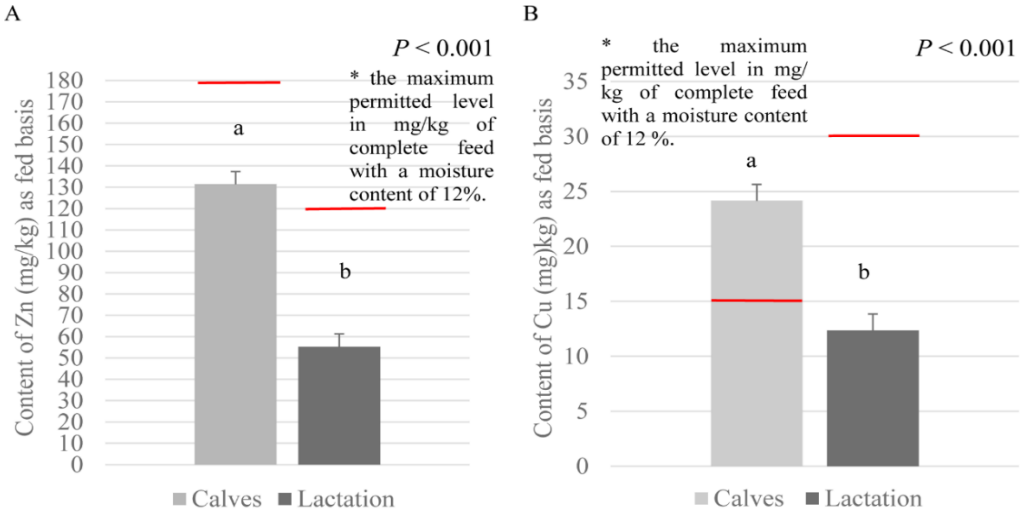
As we observed in intensive animal production systems, especially in swine feed, the common strategy is to use the maximum tolerable permitted levels of microelements such as Zn and Cu in the complete diet. This tendency is above the amount required for the animals and should not be a nutritional target. In light of those findings, it is possible to reduce the amount of excreted nutrients such as Zn and Cu in manure by (i) avoiding nutrient overformulation (ii) feeding at optimal rather than maximum permitted levels (iii) implementing a feed efficiency strategy; and (iv) controlling Zn and Cu concentrations in raw materials before creating the complete diets (Sims et al. 2005; Mendoza-Huaitalla et al. 2010; Suttle, 2010; Dumont et al. 2013).

In order to maintain the sustainable production of swine livestock, the diet formulation should be integrated into total production systems. The overall management of animal production is crucial in order to maintain animal performance and reproduction with the

minimal excretion of minerals. Moreover, feed efficiency, through advanced genetic techniques could further improve the environmental conditions and enhance the processing of feed (Sims et al. 2005; Suttle, 2010).

The data showed that the Zn and Cu concentration in cattle feed were not largely used and did not result higher than the maximum permitted level (Figure 11). In our study higher concentration of Zn and Cu was observed in the calves' phase (131.36±5.97; 15±1.48 mg/kg as fed, respectively). Compared to our results, Zhang et al. (2012) reported higher average Zn concentration in China for dairy feed in small, medium and large heard size farms (156.28; 101.78; 114.90 mg/kg DM, respectively). Moreover, the average Cu concentration in dairy feed from small and large heard size farms in China was higher than in our work (32.12; 25.98 mg/kg DM, respectively; Zhang et al. 2012). Also Dai et al. (2016) reported in USA higher average Cu concentration (37.80 mg/kg). The established thresholds (EC N° 1831/2003) for Zn and Cu in cattle feed were also not exceeded (Figure 11).

Figure 11. The average concentration of Zn content (A) in feed with the red line represents maximum permitted level of zinc as nutritional additive in feed for cattle (calves – 180 mg/kg; other – 120 mg/kg; EC N° 1831/2003, 2016/1095) and for Cu content (B) in feed with the red line represents maximum permitted level of copper as nutritional additive in feed for cattle (bovines before rumination – 15 mg/kg; other bovines – 30mg/kg; EC N° 2018/1039) from different cattle phases (calves, lactation) in considered cattle farms (F5-F8) located in northern Italy.



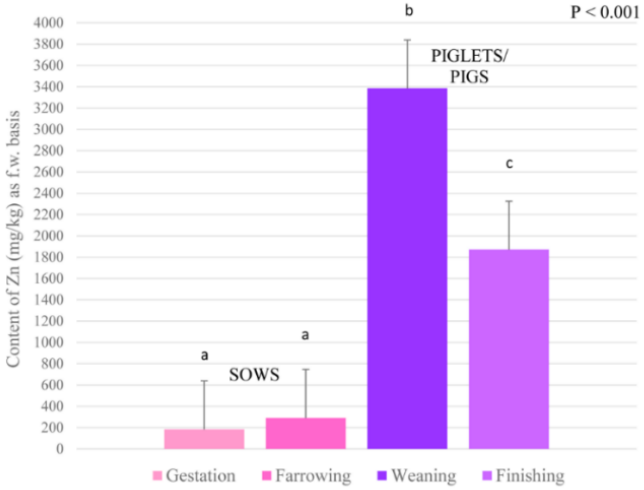
The average humidity content (% as f.w.) in cattle feed (with SE): 11.42±1.18 for calves; 49.69±3.38 for lactation. Data are presented as least squares means and SEM.

The water samples analysed regarding Zn elements from cattle farms were for F6: 0.0553 mg/L; F7: 0.2540 mg/L; F8: 0.0031 mg/L and for Cu F6: 0.0748 mg/L; F7: 0.2117 mg/L; F8: 0.0024 mg/L which did not exceed the legal limit for water (for Zn: 3 mg/L in accordance with D. Lgs. 152/2006 and for Cu: 2 mg/L in accordance with 1998/83/EC).

4.4.4. Zn and Cu content in faecal samples for swine and cattle farms

The results showed that swine faeces had a higher Zn concentration in the weaning and finishing phases in line with the same feed phases in the swine diet (Figure 12; 3385.20±454.00; 1871.60±454.00 mg/kg as f.w., respectively). In general, a wide range of Zn content was observed because many factors can altered the Zn excretion by faeces (the diet, the management, the different regulations).

Figure 12. The average concentration of Zn content (mg/kg of f.w. – fresh weight) in faeces from different swine phases (gestation, weaning, farrowing, finishing) in considered swine farms (F1-F4) located in northern Italy.



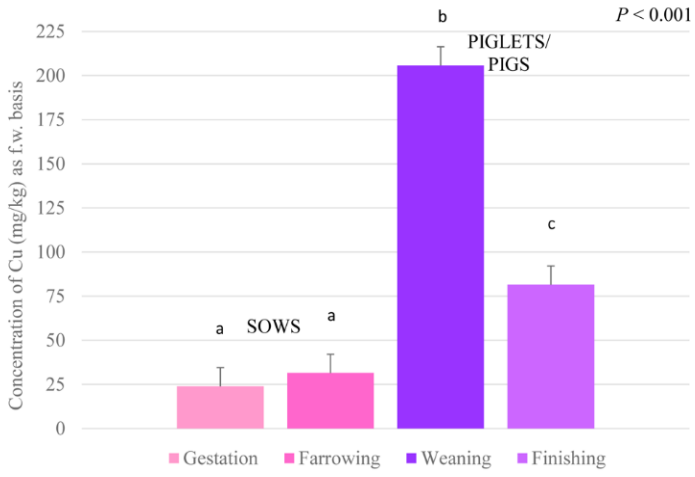
The average humidity content (% as f.w.) in swine faeces (with SE): 72.78±1.75 for gestation; 71.65±1.73 for farrowing; 70.13±2.46 for weaning; 73.52±1.10 for finishing.
Data are presented as least squares means and SEM.

Li et al. (2018) reported high average Zn concentration in the solids residue of swine manure (17467.00 mg/kg) and Xu et al. (2013) observed wide range of this element (183.42 to 1126.25 mg/kg) in pig manure in China. According to Yang et al. (2017) the range of Zn concentration in the swine compost (contain swine slurry and straw) ranged from 11.80 to 3692.00 mg kg^{-1} DW in China farms. In Europe, the situation was similar with our results and with outcomes from China. In Bavaria (Germany) Zn from swine manure ranged from 93.0 to 8239.0 mg/kg DM (Hölzel et al. 2012). In Portugal, Alvarenga et al. (2015) reported high concentration of Zn (2033.0 ±255.6 mg kg^{-1}) in pig slurry. In contrary, Nicolson et al. (2003) and Luo et al. (2009) observed the lower average Zn concentration of swine slurry in England (650.0 mg/kg DM) and in China (843.30 mg/kg). Higher concentration of Zn in swine faeces is related to the low mineral absorption from the gut. Even if the differences in concentrations of HMMs may be related to several factors the concentration of Zn and Cu in animal feed and manure were positively correlated (Wang et al. 2013). The highest Zn excretion was observed

in the weaning phase. This is probably due to the immature digestion system of the animals or by the pharmacological use of this mineral source to control the enteric disorders that can influence to the manure content (Hu et al. 2012; Liu et al. 2018). Moreover, due to the wide use of Zn at a pharmacological level, in the weaning and finishing phases, swine fed with 2,000 ppm of Zn excreted approximately 10 times more Zn in faeces than pigs fed the basal diet containing 165 ppm of Zn.

The Cu content was more concentrated in faeces compared with the feed. The average Cu concentration was the highest in the weaning phase in swine faeces (205.72±10.61 mg/kg as f.w.; Figure 13). Our results reported lower Cu concentration in faeces compared with the concentration from different literatures. The average Cu concentration in pig manure in Bavaria (Germany) ranged from 22.40 to 3387.60 mg/kg DM (Hölzel et al. 2012). Alvarenga et al. (2015) reported in Portugal high concentration of Cu (510.3±30.8 mg kg^{-1}) in a digested pig slurry. Moreover, high average Cu concentration for swine slurry was also observed in England (470.00 mg/kg DM; Nicholson et al. 2003). Furthermore, in China according to Xu et al. (2013), high average Cu concentration in pig manure was reported (418.42 mg/kg); and Yang et al. (2017) ranged the Cu concentration in the swine compost samples from 3.55 to 916.0 mg kg^{-1} DW.

Figure 13. The average concentration of Cu content (mg/kg of f.w. – fresh weight) in faeces from different swine phases (gestation, weaning, farrowing, finishing) in considered swine farms (F1-F4) located in northern Italy.



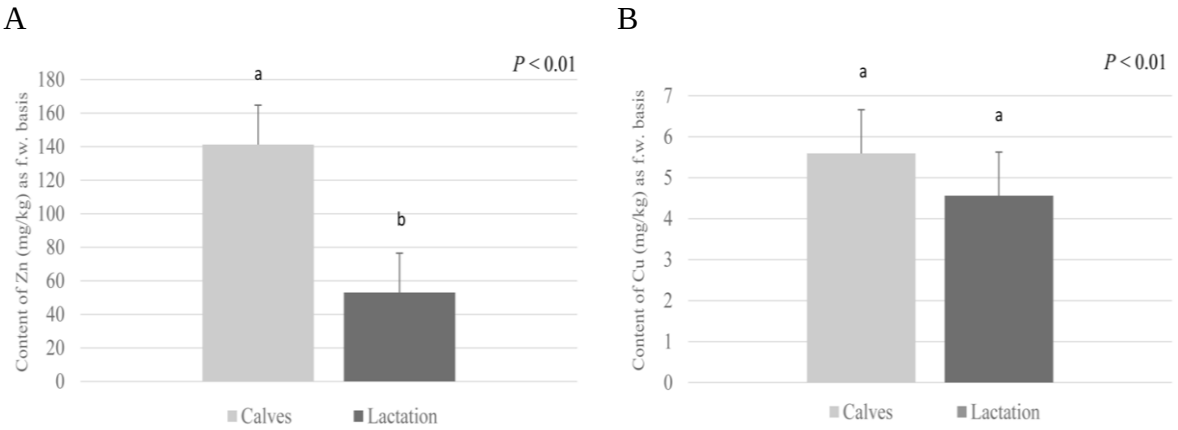
The average humidity content (% as f.w.) in swine faeces (with SE): 72.78±1.75 for gestation; 71.65±1.73 for farrowing; 70.13±2.46 for weaning; 73.52±1.10 for finishing.
Data are presented as least squares means and SEM.

Nevertheless, the Zn and Cu content in animal faeces may be altered not only by the feed input. For instance, the therapeutic treatments of animals, ingestion of the soil, livestock

bedding, hoof disinfection tanks, corrosion of containment structures, and the water could also be sources (Nicholson et al. 2003).

Our results showed that Zn and Cu were the most critical HMMs in the swine faeces and that their concentration in the manure was strictly related to the feed level. In order to increase environmental sustainability, the Zn and Cu input should be controlled considering the quality and the quantity of mineral supplements using novel additives (Bolan et al. 2003; Nicholson et al. 2003; Luo et al. 2009; Mendoza-Huaitalla et al. 2010; Zhang et al. 2012; Jakubus et al. 2013). Moreover, the different situation should be considered, because some metals are naturally present in the raw materials, which are integrated the feed diets rations. Thus, they can influence on the HMMs concentration in the feed. In line with the major topics of agroecology, considering the needs of animals (health, welfare and nutrition productivity) and farmers (profitability and productivity) together with the environment, multidisciplinary strategies about feed formulations are required. Conversely, cattle and calves' faeces did not exceed regulation thresholds in intensive animal production systems in terms of the Zn and Cu elements (Figure 14).

Figure 14. The average concentration of Zn content (A) and Cu content (B) in faeces (mg/kg of f.w. – fresh weight) from different cattle phases (calves, lactation) in considered cattle farms (F5-F8) located in northern Italy.



The average humidity content (% as f.w.) in cattle faeces (with SE): 80.03±3.12 for calves; 85.80±0.25 for lactation.
Data are presented as least squares means and SEM.

4.5. Conclusions

Our results showed that nutrition play a pivotal role in the sustainability of intensive animal rearing system. In fact, HMMs in animal manure was mainly influenced by the feed. Nevertheless, the contaminants did not result as a problem in swine and cattle diets, the most critical elements were zinc and copper probably due to their use in controlling the enteric

disorders in growing piglets. In line with agroecology principles, strategies should be adopted to reduce the flux of Zn and Cu intake from swine production In particular, the different strategies should be implemented to discover the innovative approach to control the enteric disorders. Attention should be paid to use of the additives with higher bioavailability levels consider that the raw materials can influence on total HMMs amount. Moreover, the use of optimal doses rather than maximum permitted levels of microelements should be introduced due to avoiding the nutrient overformulation.

4.6. References

[AOAC] Association of Official Analytical Chemists. 2005. Official methods of analysis. 18th ed. Gaithersburg (MD): AOAC Int.

[NRC] National research council. 2001. Nutrient requirements of dairy cattle. 7th edition. National academy press. Washington, D.C.

[NRC] National research council. 2012. Nutrient requirements of swine. 11th edition. National academy press. Washington, D.C.

Adamse P, Van der Fels-Klerx HJ, de Jong J. 2017. Cadmium, lead, mercury and arsenic in animal feed and feed materials – trend analysis of monitoring results. Food Addit Contam part A, 34, 8, 1298-1311.

Adewole DI, Kim IH, Nyachoti CM. 2016. Gut health of pigs: challenge models and response criteria with a critical analysis of the effectiveness of selected feed additives – a review. Asian Australas. J Anim Sci. 29, 909-924.

Alvarenga P, Mourinha C, Farto M, Santos T, Palma P, Sengo J, et al. 2015. Sewage sludge, compost and other representative organic wastes as agricultural soil amendments: benefits versus limiting factors. Waste Manage. 40, 44-52.

Bargo F, Muller L, Delahoy J, Cassidy T. 2002. Performance of high producing dairy cows with three different feeding systems combining pasture and total mixed rations. J Dairy Sci. 85(11), 2948-63.

Bastianelli D, Bonnal L, Jaguelin-Peyraud Y, Noblet J. 2015. Predicting feed digestibility from NIRS analysis of pig faeces. Animal. 9:5, 781-786.

Bhargava A, Carmona FF, Bhargava M, Srivastava S. 2012. Approaches for enhanced phytoextraction of heavy metals. J Environ Manage. 105, 103-120.

Bohn L, Meyer AS, Rasmussen SK. 2008. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. Zhejiang Univ Sci B. 9(3), 165-191.

Bolan NS, Khan MA, Donaldson J, Adriano DC, Matthew C. 2003. Distribution and bioavailability of copper in farm effluent. Sci Total Environ. 309, 225-236.

Chardon X, Rigolot C, Baratte C, Espagnol S, Raison C, Martin-Clouaire R, Rellier J-P, Le Gall A, Dourmad JY, Piquemal B, Leterme P, Paillat JM, Delaby L, Garcia F, Peyraud JL, Poupa JC, Morvan T, Faverdin P. 2012. MELODIE: a whole-farm model to study the dynamics of nutrients in dairy and pig farms with crops. Animal. 6:10, 1711-1721.

Commission Implementing Regulation (EU) N°2018/1039 of 23 July 2018 concerning the authorization of Copper (II) diacetate monohydrate, Copper (II) carbonate dihydroxymonohydrate, (…) as feed additive for all animal species. Off J. L: 186/3.

Commission Implementing Regulation (EU) 2016/1095 of 6 July 2016 concerning the authorization of Zinc acetate dihydrate, Zinc chloride anhydrous, Zinc oxide (…) as feed additive for all animal species. Off J. L: 182/7.

Council Directive. Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs. Off J. L: 47/5.

Council Directive. Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes. Off J. L: 221/23.

Council Directive 1998/83/EC on the quality of water intented for human consumption. OJ L 330/32.

Dai SY, Jones B, Lee K-M, Li W, Post L, Herrman TJ. 2016. Heavy metal contamination of animal feed in Texas. JRS. 01, 21-32.

Decreto legislativo 3 aprile 2006, n. 152 Norme in materia ambientale (G.U. n. 88 del 14 aprile 2006).

Dumont B, Fortun-Lamothe L, Jouven M, Thomas M, Tichit M. 2012. Prospects from agroecology and industrial ecology for animal production in the 21st century. Animal. 7:6, 1028-1043.

Eastridge ML. 2006. Major advances in applied dairy cattle nutrition. J. Dairy Sci. 89, 1311-1323.

European Commission. Commission Regulation no 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed establishes the sampling method and the methods of analysis of feed for control purposes. Off J. L 54:177.

European Medicine Agency (EMA) N° 394961/2017. Questions and answers on veterinary medicinal products containing zinc oxide to be administered orally to food-producing species.

European Parliament and of the Council. Directive 2002/32/EC of 7 May 2002 on undesirable substances in animal feed. Off J. L: 140/10.

European Parliament and the Council. Regulation (EC) no 1831/2003 of 22 September 2003 on additives for use in animal nutrition. Off J. L: 268/29.

Fink-Gremmels J. 2012. Animal Feed Contamination, 1st Edition. Effects on Livestock and Food Safety. Woodhead Publishing Limited, Cambridge.

García-Vaquero M, López-Alonso M, Benedito JL, Hernández J, Gutiérrez B, Miranda M. 2011. Influence of Cu supplementation on toxic and essential trace element status in intensive reared beef cattle. Food and Chemical Toxicology 49, 3358-3366.

Gaudré D, Quiniou N. 2009. What mineral and vitamin levels to recommend in swine diets? R Bras Zootec. v.38, 190-200.

Gul S, Naz A, Fareed I, Khan A, Irshad M. 2015. Speciation of heavy metals during co-composting of livestock manure. Pol J Chem Technol. 17 (3), 19-23.

Grandin. 2017. On-farm conditions that compromise animal welfare that can be monitored at the slaughter plant. Meat Sci. 132:52–58.

Hejna M, Gottardo D, Baldi A, Dell'Orto V, Cheli F, Zaninelli M, Rossi L. 2018. Review: Nutritional ecology of heavy metals. Animal. 1-15.

Hölzel CS, Müller C, Harms KS, Mikolajewski S, Schäfer S, Schwaiger K, et al. 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. Environ Res. 113, 21-27.

Hu C, Song J, You Z, Luan Z, Li W. 2012. Zinc Oxide-Montmorillonite hybrid influences diarrhea, intestinal mucosal integrity, and digestive enzyme activity in weaned pigs. Biol Trace Elem Res. 149, 190-196.

Indugu N, Vecchiarelli B, Baker LD, Ferguson JD, Vanamala JKP, Pitta DW. 2017. Comparison of rumen bacterial communities in dairy herds of different production. BMC Microbiology, 17:190.

Jakubus M, Dach J, Starmans D. 2013. Biovailability of copper and zinc in pig and cattle slurries. Fresen Environ Bull. Vol.22 no 4, 995-1002.

Jarup L. 2003. Hazards of heavy metal contamination. Br Med Bull. 68, 167-82.

Jha R, Berrocoso JD. 2015. Review: dietary fiber utilization and its effects on physiological functions and gut health of swine. Animal. 9, 1441-1452.

Jondreville C, Schlegel P, Hillion S, Chagneau AM and Nys Y, 2007. Effects of additional zinc and phytase on zinc availability in piglets and chicks fed diets containing different amounts of phytates. Livestock Science. 109, 60-62.

Le Goff G, Noblet J. 2001. Comparative total tract digestibility of dietary energy and nutrients in growing pigs and adult sows. J Anim Sci. 79, 2418-2427.

Leonardi C, Giannico F, Armentano LE. 2005. Effect of water addition on selective consumption (sorting) of dry diets by dairy cattle. J. Dairy Sci. 88, 1043-1049.

Li H, Lu J, Zhang Y, Liu Z. 2018. Hydrothermal liquefaction of typical livestock manures in China: Biocrude oil production and migration of heavy metals. J Anal Appl Pyrolysis. 135,133-140.

Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, Kwon WB, Mathai JK, Navarro DMDL, Jaworski NW, Stein HH. 2018. Non-antibiotic feed additives in diets for pigs: A review. Animal Nutrition. 1-13.

Lopez-Alonso M, Garcia-Vaquero M, Benedito JL, Castillo C, Miranda M. 2012. Trace mineral status and toxic metal accumulation in extensive and intensive pigs in NW Spain Livest Sci. 146, 47-53.

Luo L, Maa Y, Zhang S, Wei D, Zhu Y-G. 2009. An inventory of trace element inputs to agricultural soils in China. J Environ Manage. 90, 2524-2530.

Lyubenova L, Schröder P. 2011. Plants for waste water treatment – Effects of heavy metals on the detoxification system of *Typha latifolia*. Bioresour Technol. 102, 996-1004.

Mendoza-Huaitalla R, Gallmann E, Zheng K, Liu X, Hartung E. 2010. Heavy metals contents in farrowing, weaning and fattening pig feeds in a commercial pig farm in Beijing and their thresholds values given by the Chinese feed standards. Advances in Animal Biosciences. Vol 1 (2), 470-471.

Miller EL. 2004. *Protein nutrition requirements of farmed livestock and dietary supply* in Protein sources for the animal feed industry. Food and agriculture organization of the United Nations. Rome 2004.

Moral R, Moreno-Caselles J, Perez-Murcia MD, Perez-Espinosa A, Rufete B, Paredes C. 2005. Characterisation of the organic matter pool in manures. Bioresour Technol. 96, 153-158.

Nicholson FA, Chambers BJ, Williams JR, Unwin RJ. 1999. Heavy metal contents of livestock feeds and animal manures in England and Wales. Bioresour Technol. 70, 23-31.

Nicholson FA, Smith SR, Allowayc BJ, Carlton-Smithd C, Chambersa BJ. 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. Sci Total Environ. 311, 205-219.

Patience JF, Rossoni-Serão MC, Gutiérrez NA. 2015. A review of feed efficiency in swine: biology and application. J Anim Sci Biotechnol. 6-33.

Pearce SC, Sanz Fernandez MV, Torrison J, Wilson ME, Baumgard LH, Gabler NK. 2015. Dietary organic zinc attenuates heat stress-induced changes in pig intestinal integrity and metabolism. J Anim Sci. 93, 4702-13.

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

Rossi L, Fusi E, Boglioni M, Giromini C, Rebucci R, Baldi A. 2014b. Effect of Zinc oxide and Zinc chloride on human and swine intestinal epithelial cell lines. International Journal of Health, HAF. 2, 1-7.

Rossi L, Dell'Orto V, Vagni S, Sala V, Reggi S, Baldi A. 2014a. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. Vet Res Commun. 38(1), 39-49.

Shi T, Ma J, Wu F, Ju T, Gong Y, Zhang Y, et al. 2019. Mass balance-based inventory of heavy metals inputs to and outputs from agricultural soils in Zhejiang Province, China. Sci Total Environ. 649, 1269-1280.

Sims JT, Bergstro L, Bowman BT, Oenema O. 2005. Nutrient management for intensive animal agriculture: policies and practices for sustainability. Soil Use and Manage. 21, 141-151.

Suttle NF. 2010. Mineral Nutrition of Livestock, 4th Edition. Cabi, Oxfordshire.

Terré M, Pedrals E, Dalmau A, Bach A. 2013. What do preweaned and weaned calves need in the diet: A high fiber content or a forage source? J. Dairy Sci. 96, 5217-5225.

Van Vliet PCJ, Reijs JW, Bloem J, Dijkstra J, de Goede RGD. 2007. Effects of cow diet on the microbial community and organic matter and nitrogen content of feces. J. Dairy Sci. 90, 5146-5158.

Wang H, Dong Y, Yang YS, Toor G, Zhang X. 2013. Changes in heavy metal contents in animal feeds and manures in an intensive animal production region of China. J Environ Sci (China). 25(12), 2435-2442.

Wang LF, Zhang H, Beltranena E, Zijlstra RT. 2018. Diet nutrient and energy digestibility and growth performance of weaned pigs fed hulled or hull-less barley differing in fermentable starch and fibre to replace wheat grain. Anim Feed Sci Technol. 242, 59-68.

Warly L, Evitayani S, Fariani A. 2017. Nutrient digestibility and apparent bioavailability of minerals in beef cattle fed with different levels of concentrate and oil-palm fronds. Pak. J. Nutr.16 (3), 131-135.

Xu Y, Yu W, Ma Q, Zhou H. 2013. Accumulation of copper and zinc in soil and plant within ten-year application of different pig manure rates. Plant Soil Environ. Vol. 59, No. 11, 492-499.

Zhang B, Guo Y. 2009. Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. Br J Nutr. 102, 687-93.

Zhang F, Li Y, Yang M, Li W. 2012. Content of heavy metals in animal feeds and manures from farms of different scales in northeast China. Int J Environ Res Public Health. 9, 2658-2668.

Yang X, Li Q, Tang Z, Zhang W, Yu G, Shen Q, et al. 2017. Heavy metal concentrations and arsenic speciation in animal manure composts in China. Waste Manage. 64, 333-339.

CHAPTER 4

Brief introduction of the scientific works of evaluation of plant-based compounds on weaned pigs health and performance

The current situation in intensive food-producing animals related to antimicrobial resistance and potential source of heavy metals output from livestock wastewater to the environment is significant global concern. The first adopted alternative against in feed antibiotics was the wide application of high doses of essential nutrients such as zinc and copper salts in the form of premix raised many concerns related to environmental pollution. Hence, in order to reduce input, innovative in-feed plant based extracts and phytochemicals are required to prevent enteric disorders during the weaning and to enhance the gut health. One of the alternative may be natural plant-based phytochemicals such as tannins, leonardite or mint oils which are widely studied because of their antimicrobial, antioxidant and anti-inflammatory properties. This chapter thus focused on (i) evaluation the *in vivo* effect of tannin in order to establish if the inclusion of 1.25% combined chestnut and quebracho tannins can induce a positive effect on weaned piglets, (ii) evaluation of the effect of leonardite included at 0.25%, as natural material rich in HAs, on the principal metabolic parameters and growth of weaned piglets and (iii) evaluation of the *in vitro* anti-inflammatory effects of peppermint oil and spearmint oil with porcine alveolar macrophages as host immune responses. The experiment with peppermint oil and spearmint oil was developed in collaboration with University of California in Davis (USA) where I spend my period abroad to gain an international experience.

Experiment 2:

Evaluation of dietary administration of chestnut and quebracho tannins on growth, serum metabolites and faecal parameters of weaned piglets

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5.1. Abstract

In pig livestock, alternatives to in-feed antibiotics are needed to control enteric infections. Plant extracts such as tannins can represent an alternative as a natural source of functional compounds. The aim of this study was to evaluate the *in vitro* digestibility and *in vivo* effects of oral supplementation of combined chestnut (Ch) and quebracho (Qu) tannins in order to establish if they can induce a positive effect on weaned piglets' performance, metabolic status and fecal parameters. *In vitro* digestibility (dry matter, DM) of diets was calculated using a multi-step enzymatic technique. *In vitro* digested diet samples were further tested on an intestinal porcine enterocyte cell line (IPEC-J2). Weaned piglets (n = 120; 28 ± 2 day old) were randomly allotted to two groups (12 pens in total with 10 pigs per pen): control (Ctrl) and treatment (Ch/Qu). After one week of adaptation (day 0), 35-day-old piglets in the Ctrl group were fed a Ctrl diet and the Ch/Qu group were fed with 1.25% Ch/Qu for 40 days. Body weight and feed intake per pen were recorded weekly. At day 40, blood and fecal samples were collected. Principal metabolic parameters were evaluated from blood samples by enzymatic colorimetric analysis. Total phenolic compounds, urea, and ammonia in faeces were analyzed (Megazyme International, Bray, Ireland). *In vitro* digestibility and cell viability assays showed that the inclusion of 1.25% Ch/Qu slightly reduced diet digestibility compared with the Ctrl diet, while intestinal cell viability was not altered with low concentrations of Ch/Qu digesta compared with Ctrl. *In vivo* results did not show any adverse effects of Ch/Qu on feed intake and growth performance, confirming that dietary inclusion of Ch/Qu at a concentration of 1.25% did not impair animal performance. The decreased diet DM digestibility in the Ch/Qu diet may cause increased serum concentration of albumin (Ctrl: 19.30 ± 0.88; Ch/Qu: 23.05 ± 0.88) and albumin/globulin ratio (Ctrl: 0.58 ± 0.04; Ch/Qu: 0.82 ± 0.04), but decreased creatinine (Ctrl: 78.92 ± 4.18; Ch/Qu: 54.82 ± 4.18) and urea (Ctrl: 2.18 ± 0.19; Ch/Qu: 0.95 ± 0.19) compared with Ctrl. Pigs in the Ch/Qu group contained higher (p < 0.05) concentrations of fecal phenolic compounds and nitrogen than the Ctrl group, while fecal ammonia and urea were not affected by tannins. In conclusion, Ch/Qu tannin supplementation did not influence growth performance. Although lower digestibility was observed in the diet supplemented with Ch/Qu tannins, Ch/Qu supplementation did not show any adverse effect on intestinal epithelial cell viability.

5.2. Introduction

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

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

Due to such restrictions, alternatives to antibiotics and alternatives to zinc oxide are urgently needed to guarantee animal production in line with health principles [11,12]. In this scenario, plant extracts can represent a valuable alternative as a natural source of functional compounds, such as polyphenolic compounds [13–17]. Among polyphenols, tannins derived from plant extracts are the largest class which can be classified into hydrolysable (HTs) or condensed (CTs) subgroups [18]. Tannins have been tested in the poultry and swine sectors, initially as tannin-rich feedstuffs (such as sorghum, barley, maize and fava beans), and more recently as tannin extracts from different plants (grape seed, grape pomace, acorns, oak, green tea leaves, and pomegranate) [19,20]. Chestnut trees (Ch, *Castanea sativa* Mill.) are a source of HTs, whereas quebracho trees (Qu, *Schinopsis* spp.) are a source of CTs or proanthocyanidins [21]. Tannins extracted from these plants have been applied in intensive swine farms due to their antioxidant, anti-inflammatory, and antibacterial activities [16,21].

Heterogeneous results were obtained on the effects of Ch and Qu to enhance growth performance, modulate intestinal microbiota, and decrease the incidence of diarrhoea during the post-weaning period [22–24]. These heterogeneous results could be related to the chemical characteristics of tannins, which can compromise the palatability, digestibility, and protein use of feed. The ability to bind proteins and carbohydrates in monogastric animals is associated with the antinutritional effects of tannins in reducing feed palatability [18]. Thus, contrasting results on the effective supplementation of tannins on pigs' performance and intestinal health

have been observed in relation to the source of tannins (Ch and Qu), dosage of tannins, and the type of tannins (HTs or CTs) included in the diets [25,26]. The heterogeneity of commercial products is associated with the use of Ch or Qu individually or in mixtures with different percentages of tannins (from 54% to 82%) and hence different amounts of HTs and CTs.

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

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

5.3. Materials and Methods

5.3.1. Animals, housing, experimental design and treatment

Our *in vivo* trial complied with Italian regulations on animal experimentation and ethics (DL 26/2014) [30] in accordance with European regulation (Dir. 2010/6) [31] and was approved by the Animal Welfare Body of the University of Milan (number 31/2019). This trial was performed in an intensive conventional herd farm located in Lombardy (Italy) that was free from any of the diseases reported in the previous A-list of the International Office of Epizootics, and free from Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome, and salmonellosis. A total of 120 crossbred piglets (Large White × Landrace), weaned at 28 ± 2 days (50% female and 50% male), were identified using individual ear tags and allotted in randomized complete block design into two experimental groups: control group (Ctrl) and treatment group (Ch/Qu). There were 60 pigs per treatment with 6 replicate pens and 10 pigs per pen. The groups were homogeneous in terms of gender, weight and litter. After one week of adaptation (considered day 0, piglets were 35 days old), during which all animals received the same basal diet, the experimental diets were distributed ad libitum to all animals for 40 days. Experimental diets (Plurimix, Fabermatica, CR, Italy) were formulated according to animal requirements for the post-weaning phase

(Ferraroni Mangimi SpA, Bonemerse, Italy). The two diets were isoenergetic and isoproteic and fulfilled the NRC (2012) [32] requirements for post-weaned piglets (Table 15). The Ch/Qu diet was differentiable by the inclusion of 1.25% of tannin extract from chestnut and quebracho trees (Silvafeed for Swine, Silvateam, Italy).

Table 15. Ingredient composition of the experimental diets administered to weaned piglets (control (Ctrl), *n* = 60; chestnut/quebracho (Ch/Qu), *n* = 60) from day 0 to day 40 of the experimental trial on an as-fed basis.

Ingredients [1,2,3,4,5], as % of Fed Basis	**Ctrl**	**Ch/Qu**
Barley meal	25.15	25.00
Wheat meal	19.41	19.07
Corn meal	14.03	13.50
Corn flakes	4.85	4.80
Soybean meal	4.65	4.60
Soybean protein	4.11	4.10
Bakery meal	4.00	4.00
Dextrose monohydrate	3.50	3.50
Wheat middlings	4.32	4.30
Fermented milk product	3.00	3.00
Fish meal	2.50	2.50
Milk whey powder	2.50	2.50
Coconut oil	1.00	1.00
Soy oil	1.00	1.00
Plasma, meal	1.00	1.00
Dicalcium phosphate	0.85	0.80
Animal fats, lard	0.70	0.70
L-Lysine	0.50	0.50
Acidity regulators[5]	1.00	1.00
Benzoic acid	0.40	0.40
L-Threonine	0.34	0.34
DL-Methionine	0.35	0.35
Sodium chloride	0.26	0.24
Vitamins	0.24	0.24
L-Valine (96.5%)	0.14	0.14
L-Tryptophan	0.08	0.05
Copper sulfate	0.04	0.04
Ch/Qu Tannins [6]	-	1.25

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

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

5.3.2. Chemical analyses and in vitro digestibility evaluation of Ctrl and Ch/Qu diets

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

Total phenolic compounds in Ctrl and Ch/Qu diets and in Ch/Qu tannin extracts were assayed according to the Folin-Ciocalteu method [37]. Each feed sample was weighed (5 ± 0.5 g) and mixed with 30 mL of pure methanol (Sigma Chemical Co, St. Louis, MO, USA) for 24 h at room temperature and subsequently filtered (Whatman 54, Florham Park, NJ, USA). The obtained chemical extracts from feed samples were tested for total phenolic compounds. Prior to the analysis, a standard curve was prepared using tannic acid (Sigma Chemical Co, St. Louis, MO, USA). The tannic acid was water-dissolved to obtain a stock solution of 960 μg/mL. Dilutions of the stock solution were prepared to obtain final concentrations from 60 to 960 μg tannic acid/mL. The Folin-Ciocalteu reagent was diluted 1:10 with deionized water and a solution of 1 M sodium carbonate (Sigma Chemical Co, St. Louis, MO, USA) was prepared. Briefly, an aliquot (0.5 mL) of extract, blank or standard was placed in a 15 mL plastic tube, where the Folin-Ciocalteu reagent (2.5 mL) and sodium carbonate (2 mL) were added and the mixture was incubated at room temperature in a dark chamber for 20 min. The total phenolic content was determined by colorimetry at 630 nm using an UV-visible spectrophotometer (Jasco V-630, Easton, MD, USA). Total phenolic content was expressed as tannic acid equivalents (g TAE/kg). The analyses were performed in technical duplicate and biological triplicate.

The Ctrl and Ch/Qu diets adopted in the *in vivo* trial were *in vitro* digested using the protocol reported by Reggi et al. [16]. The *in vitro* digestion was performed according to the protocol described by Regmi et al. [38] with minor adaptations previously reported by our group [39]. At the end of the *in vitro* digestion procedure, a soluble fraction and an undigested fraction (UF) were obtained. The soluble fraction was used for cell viability assays (detailed below).

The UF was then collected in a filtration unit using a porcelain filtration funnel lined with pre-weighed filter paper (Whatman no. 54). The UF, along with the filter paper, were dried overnight at 65 °C. The UF was used to calculate the *in vitro* digestibility (IVD) using Equation (1):

$$\text{IVD (\%)} = (\text{sample (DM)} - \text{sample UF (DM)})/(\text{sample (DM)} \times 100). \quad (1)$$

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

5.3.3. Effect of Ctrl and Ch/Qu diet on swine intestinal cell viability

The intestinal porcine enterocyte cell line IPEC-J2 is unique as it is derived from the small intestine isolated from the jejunum of a neonatal unsuckled piglet (ACC 701, DSMZ, Braunschweig, Germany) and is not transformed nor tumorigenic in nature [39]. IPEC-J2 cells were cultured in Dulbecco's Modified Eagle Medium + Ham's F-12 mixture (DMEM/F-12) supplemented with HEPES (N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)), fetal bovine serum (FBS), penicillin/ streptomycin and cultivated in a humid chamber at 37 °C with 5% CO_2. All experiments were performed using IPEC-J2 cells within six cell passages (passages 16 to 22) to ensure reproducibility. In particular, IPEC-J2 cells were seeded at a density of 1.5–2 × 10^5 cells/mL in 96-well plates and cultured for 24 h.

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

5.3.4. Collection of fecal and blood samples

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

(relative centrifugal force) for 10 min at 4 °C. Serum was aliquot and stored at −20 °C for further analysis.

5.3.5. Zootechnical evaluation

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

5.3.6. Total phenolic compounds, urea and ammonia in faeces

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

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

5.3.7. Blood serum analysis

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

5.3.8. Statistical analysis

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

5.4. Results

5.4.1. Chemical analyses and in vitro digestibility evaluation of experimental Ch/Qu and Ctrl diets

The experimental diets contained a similar content of principal nutrients (Table 16). The phenolic content differed due to the tannin supplementation, and was 3.68 times higher in

Ch/Qu group than in the Ctrl group. The *in vitro* experiments showed a slight reduction of DM digestibility in the Ch/Qu diet (69.33% of DM) compared to the Ctrl diet (72.00% of DM).

Table 16. Chemical analysis of experimental diets fed to piglets from day 0 to day 40 [1] of the *in vivo* trial (from 35- to 75-day-old piglets).

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

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

5.4.2. Swine intestinal cell viability

Samples of *in vitro* digested Ch/Qu and control diets were tested on IPEC-J2 cell viability at diluted concentrations. In general, Ch/Qu and Ctrl digesta showed comparable effects on cell viability at the tested concentrations. An exception was represented by the concentration of 5.32 mg/mL of digesta. At this concentration, Ch/Qu seems to detrimentally affect viability compared to Ctrl. In contrast, at a concentration of 1.33 mg/mL, Ch/Qu promoted cell viability compared to Ctrl ($p < 0.05$) (Figure 15).

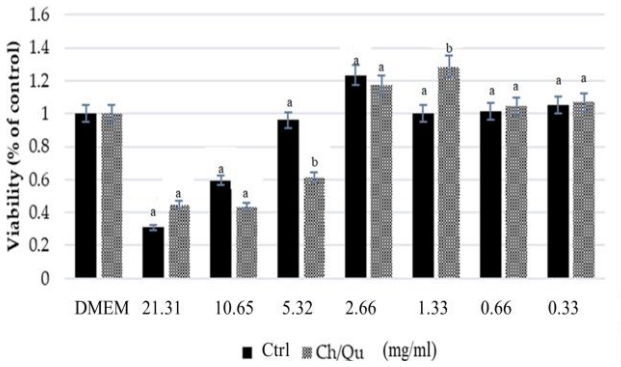

Figure 15. Effect of different concentrations (21.31–0.33 mg/mL) of Ctrl and Ch/Qu digesta (<3 kDa) on intestinal porcine epithelial cell line IPEC-J2 cell viability (via 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide MTT assay). Data are expressed as percentage of control (DMEM - Dulbecco's Modified Eagle Medium + Ham's F-12 mixture, 0 mg/mL) as least squares means (LSMEANS) and standard errors of the means (SEM) (n = 3). At each concentration, different letters denote statistical differences between Ch/Qu and Ctrl.

5.4.3. Zootechnical performance

No mortality and no veterinary interventions were observed in the Ch/Qu and Ctrl groups during the entire experimental period. Moreover, no differences between males and females were observed in the analyzed parameters. Tannin supplementation did not affect ADFI, BW,

ADG or feed-to-gain ratio (Table 17). Daily phenolic compound intake was significantly higher ($p < 0.01$) in the Ch/Qu group compared to the Ctrl group during the entire experimental period.

Table 17. Growth performance of weaned piglets fed diets with tannins (Ch/Qu, $n = 60$) or without supplementation (Ctrl, $n = 60$) from day 0 to day 40 [1] of the *in vivo* trial (from 35- to 75-day-old piglets).

Growth performance	Treatments [2]		SEM	*p*-Value		
	Ctrl	Ch/Qu		Treatment	Day	Trt × Day
Phenolic compound intake, g/d	0.656 [a]	2.341 [b]	0.15	<0.01	<0.01	<0.01
ADFI, kg/d	0.623	0.599	0.02	0.258	<0.01	0.241
BW, kg	13.86	13.64	0.64	0.819	<0.01	0.987
ADG, kg/d	0.292	0.284	0.02	0.729	<0.01	0.651
Feed:Gain, d	1.98	2.04	0.07	0.510	0.040	0.140

[a,b] Indicate differences between treatment groups at $p < 0.05$ within the same day (Trt × Day: $p < 0.05$). [1] Data are shown as LSMEANS and SEM. [2] Ctrl: basal diet; Ch/Qu: basal diet with tannins (1.25%). ADFI: average daily feed intake; BW: body weight; ADG: average daily gain.
Regarding the health status of the animals throughout the experimental period in both groups, clinical signs of diarrhoea occurred after seven days and the signs were transient (on average lasting for three days). The occurrence of diarrhoea (number of new animals with signs of diarrhoea per group) was 3.39% and 5.00% in the Ctrl and Ch/Qu groups at day 0-14, respectively; at day 14-28, the incidence was 18.64% and 15.52% in the Ctrl and Ch/Qu groups; and at day 28–40, the incidence was 1.69% and 3.45% in the Ctrl and Ch/Qu groups.

5.4.4. Blood serum metabolites

Dietary treatment with tannins did not influence total protein content, globulin, ALT-GPT, AST-GOT, ALP, glucose, total bilirubin, total cholesterol, triglycerides, HDL, LDL, calcium, phosphorus or magnesium (Table 18). Pigs in the Ch/Qu group had higher albumin ($p = 0.006$) and A/G ratio ($p = 0.001$), but lower creatinine ($p < 0.001$) and urea ($p = 0.001$) compared with pigs in the Ctrl group.

Table 18. The biochemical concentration of serum metabolites in control (Ctrl, $n = 12$) and tannin (Ch/Qu, $n = 12$) groups on day 40 of the *in vivo* trial (75-day-old piglets) with reference ranges for pigs.

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

Total cholesterol, mmol/L	2.51	2.38	0.13	0.479	1.3–4.2 [41–43]
Triglycerides, mmol/L	0.68	0.58	0.06	0.259	0.3–2.7 [41]
HDL, mmol/L	0.77	0.75	0.03	0.667	-
LDL, mmol/L	1.60	1.52	0.10	0.532	-
Calcium, mmol/L	2.28	2.29	0.06	0.923	2.02–3.21[42]
Phosphorus, mmol/L	3.05	3.02	0.08	0.774	1.46–3.45 [42]
Magnesium, mmol/L	0.85	0.80	0.02	0.214	0.9–1.2 [41]

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

5.4.5. Influence of dietary treatment with tannins on faeces

Weaned piglets in the Ch/Qu group showed higher concentrations of fecal phenolic compounds than the Ctrl group ($p = 0.047$). Fecal nitrogen concentrations were significantly higher ($p = 0.002$) in the Ch/Qu group than the Ctrl group, while fecal ammonia ($p = 0.684$) and urea ($p = 0.235$) were not affected by the dietary inclusion of tannins (Table 19).

Table 19. Fecal metabolites of weaned piglets fed diets with tannins (Ch/Qu, $n = 6$) or without tannin supplementation (Ctrl, $n = 6$) on day 40 [1] of the *in vivo* trial (from 35- to 75-day-old piglets).

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

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

5.4.6. Influence of dietary treatment with tannins on correlation among feed, faeces and blood parameters

We found a significantly positive correlation between fecal nitrogen and phenolic compound intake ($p = 0.007$). There was also a negative correlation between fecal nitrogen and blood urea (Table 20).

Table 20. Correlation (Spearman correlation) among feed, faeces ($n = 6$/treatment) and blood parameters ($n = 6$/treatment) on day 40 [1,2] of the *in vivo* trial (from 35- to 75-day-old piglets).

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

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

5.5. Discussion

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

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

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

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

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

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

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

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

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

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

5.6. Conclusions

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

5.7. References

1. Rossi, L.; Vagni, S.; Polidori, C.; Alborali, G.L.; Baldi, A.; Dell'Orto, V. Experimental induction of *Escherichia coli* diarrhoea in weaned piglets. *J. Vet. Med.* **2012**, *2*, 1–8, doi: 10.4236/ojvm.2012.21001.
2. Rossi, L.; Di Giancamillo, A.; Reggi, S.; Domeneghini, C.; Baldi, A.; Sala, V.; Dell'Orto, V.; Coddens, A.; Cox, E.; Fogher, C. Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. *J. Vet. Sci.* **2013**, *14*, 263–270, doi:10.4142/jvs.2013.14.3.263.
3. Rossi, L.; Pinotti, L.; Agazzi, A.; Dell'Orto, V.; Baldi, A. Plant bioreactors for the antigenic hook-associated flgK protein expression. *Ital. J. Anim. Sci.* **2014**, *13*, 2939, doi: 10.4081/ijas.2014.2939.
4. Rossi, L.; Dell'Orto, V.; Vagni, S.; Sala, V.; Reggi, S.; Baldi, A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. *Vet. Res. Commun.* **2014**, *38*, 39–49, doi:10.1007/s11259-013-9583-9.
5. EU Directive 1831/2003 of the European Parliament and of The Council on additives for use in animal nutrition. *Off. J. Eur. Union* **2003**, 268, 29-43.
6. Cromwell, G. Why and how antibiotics are used in swine production. *Anim. Biotechnol.* **2002**, *13*, 7–27, doi:10.1081/ABIO-120005767.
7. Hejna, M.; Gottardo, D.; Baldi, A.; Dell'Orto, V.; Cheli, F.; Zaninelli, M.; Rossi, L. Review: Nutritional ecology of heavy metals. *Animal* **2018**, *12*, 2156–2170, doi:10.1017/S175173111700355X.
8. Hejna, M.; Moscatelli, A.; Stroppa, N.; Onelli, E.; Pilu, S.; Baldi, A.; Rossi, L. Bioaccumulation of heavy metals from wastewater through a *Typha latifolia* and *Thelypteris palustris* phytoremediation system. *Chemosphere* **2020**, *241*, 125018, doi: 10.1016/j.chemosphere.2019.125018.
9. Hejna, M.; Moscatelli, A.; Onelli, E.; Baldi, A.; Pilu, S.; Rossi, L. Evaluation of concentration of heavy metals in animal rearing system. *Ital. J. Anim. Sci.* **2019**, *18*, 1372–1384, doi: 10.1080/1828051X.2019.1642806.
10. European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), & European Medicines Agency (EMA). ECDC/EFSA/EMA Second Joint Report on the Integrated Analysis of the Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Humans and Food-Producing Animals: Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA J.* **2017**, *15*, e04872.
11. Sotira, S.; Dell'Anno, M.; Caprarulo, V.; Hejna, M.; Pirrone, F.; Callegari, M.L.; Tucci, T.V.; Rossi, L. Effects of tributyrin supplementation on growth performance, insulin, blood metabolites and gut microbiota in weaned piglets. *Animals* **2020**, *10*, 726, doi: 10.3390/ani10040726.
12. Dell'Anno, M.; Hejna, M.; Sotira, S.; Caprarulo, V.; Reggi, S.; Pilu, R.; Miragoli, F.; Callegari, M.L.; Panseri, S.; Rossi, L. Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets. *Anim. Feed Sci. Tech.* **2020**, *266*, 114519, doi.org/10.1016/j.anifeedsci.2020.114519.
13. Onelli, E.; Moscatelli, A.; Gagliardi, A.; Zaninelli, M.; Bini, L.; Baldi, A.; Caccianiga, M.; Reggi, S.; Rossi, L. Retarded germination of *Nicotiana tabacum* seeds following insertion of exogenous DNA mimics the seed persistent behavior. *PLoS ONE* **2017**, *12*, e0187929, doi:10.1371/journal.pone.0187929.
14. Liu, Y.; Song, M.; Che, T.M.; Almeida, J.A.S.; Lee, J.J.; Bravo, D.; Maddox, C.W.; Pettigrew, J.E. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic *Escherichia coli*. *J. Anim. Sci.* **2013**, *91*, 5294–5306, doi: 10.2527/jas.2012-6194.

15. Dell'Anno, M.; Sotira, S.; Rebucci, R.; Reggi, S.; Castiglioni, B.; Rossi, L. *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts. *Ital. J. Anim. Sci.* **2020**, *19*, 103–113, doi: /10.1080/1828051X.2019.1703563.
16. Reggi, S.; Giromini, C.; Dell'Anno, M.; Baldi, A.; Rebucci, R.; Rossi, L. *In vitro* digestion of chestnut and quebracho tannin extracts: Antimicrobial effect, antioxidant capacity and cytomodulatory activity in swine intestinal IPEC-J2 cells. *Animals* **2020**, *10*, 195, doi: 10.3390/ani10020195.
17. Giromini, C.; Lovegrove, J.A.; Givens, D.I.; Rebucci, R.; Pinotti, L.; Maffioli, E.; Tedeschi, G.; Sundaram, T.S.; Baldi, A. *In vitro*-digested milk proteins: Evaluation of angiotensin-1-converting enzyme inhibitory and antioxidant activities, peptidomic profile, and mucin gene expression in HT29-MTX cells. *J. Dairy Sci.* **2019**, *102*, 10760–10771, doi: 10.3168/jds.2019-16833.
18. Huang, Q.; Liu, X.; Zhao, G.; Hu, T.; Wang, Y. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Anim. Nutr.* **2018**, *4*, 137–150, 10.1016/j.aninu.2017.09.004.
19. Cappai, M.G.; Wolf, P.; Pinna, W.; Kamphues, J. Dose-response relationship between dietary polyphenols from acorns and parotid gland hypertrophy in pigs. *Food. Nutr. Sci.* **2012**, *3*, 1261, doi: 10.4236/fns.2012.39166.
20. Hao, R.; Li, Q.; Zhao, J.; Li, H.; Wang, W.; Gao, J. Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets. *Livest. Sci.* **2015**, *178*, 237–242, doi:10.1016/j.livsci.2015.06.004.
21. Girard, M.; Bee, G. Invited review: Tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. *Animal* **2020**, *14*, 95–107, doi: 10.1017/S1751731119002143.
22. Biagia, G.; Cipollini, I.; Paulicks, B.R.; Roth, F.X. Effect of tannins on growth performance and intestinal ecosystem in weaned piglets. *Arch. Anim. Nutr.* **2010**, *64*, 121–135, doi: 10.1080/17450390903461584.
23. Caprarulo, V.; Giromini, C.; Rossi, L. Review: Chestnut and quebracho tannins in pig nutrition: A review of the effects on performance and intestinal health. *Animal* **2020**. (in press)
24. Girard, M.; Hu, D.; Pradervand, N.; Neuenschwander, S.; Bee, G. Chestnut extract but not sodium salicylate decrease the severity of diarrhea and enterotoxigenic *Escherichia coli* F4 shedding in artificially infected piglets. *BioRxiv* **2019**, 15(2), 0214267. , doi: 10.1371/journal.pone.0214267.
25. Bilic-Sobot, D.; Kubale, V.; Skrlep, M.; Candek-Potokar, M.; Povse, M.; Fazarinc, G.; Skorjanc, D. Effect of hydrolysable tannins on intestinal morphology, proliferation and apoptosis in entire male pigs. *Arch. Anim. Nutr.* **2016**, *70*, 378–388, doi:10.1080/1745039X.2016.1206735.
26. Candek-Potokar, M.; Skrlep, M.; Lukac, N.; Zamaratskaia, G.; Povse, M.; Bolta, S.; Kubale, V.; Bee, G. Hydrolysable tannin fed to entire male pigs affects intestinal production, tissue deposition and hepatic clearance of skatole. *Vet. J.* **2015**, *204*, 162–167, doi:10.1016/j.tvjl.2015.02.012.
27. Brus, M.; Bavdek, S.V.; Skok, J.; Škorjanc, D. Impact of supplementing pig diet with tannins on histological characteristics of small intestine and growth performance of fattening pigs. *Acta Agric. Slov. Suppl.* **2013**, *4*, 135–138.
28. Girard, M.; Thanner, S.; Pradervand, N.; Hu, D.; Ollagnier, C.; Bee, G. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental ETEC F4 model. *PLoS ONE* **2018**, *13*, e0197878, doi:10.1371/journal.pone.0197878.
29. Tretola, M.; Maghin, F.; Silacci, P.; Ampuero, S.; Bee, G. Effect of supplementing hydrolysable tannins to a grower–finisher diet containing divergent PUFA levels on growth

performance, boar taint levels in back fat and intestinal microbiota of entire males. *Animals* **2019**, *9*, 1063, doi: 10.3390/ani9121063.
30. EU. Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. *Off. J. Eur. Union Official* **2010**, L276, 33–79.
31. EU. Commission Directive 2010/6/EU of 9 February 2010 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards mercury, free gossypol, nitrites and Mowrah, Bassia, Madhuca. *Off. J. the Eur. Union* **2010**, *37*, 29–32.
32. *National Research Council*, *Nutrient Requirements of Swine*; National Academies Press: **2012.** Washington D.C.
33. EU Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs. *Off. J. Eur. Union* **2008**, *316*, 36–38.
34. EU Commission Regulation 152/2009 laying down the methods of sampling and analysis for the official control of feed. *Off. J. the Eur. Union.* **2009,** *54*, 1-130.
35. International Organization for Standardization. *ISO-24333: Cereal and Cereal Products—Sampling*; International Organization for Standardization: Geneva, Switzerland, 2009.
36. AOAC. *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Washington, DC, USA, 2005.
37. Attard, E.A. Rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. *Open Life Sci.* **2013**, *8*, 48–53.
38. Regmi, P.R.; Ferguson, N.S.; Zijlstra, R.T. *In vitro* digestibility techniques to predict apparent total tract energy digestibility of wheat in grower pigs. *J. Anim. Sci.* **2009**, *87*, 3620–3629, doi: 10.2527/jas.2008-1739.
39. Castrica, M.; Rebucci, R.; Giromini, C.; Tretola, M.; Cattaneo, D.; Baldi, A. Total phenolic content and antioxidant capacity of agri-food waste and by-products. *Ital. J. Anim. Sci.* **2019**, *18*, 336–341, doi:10.1080/1828051X.2018.1529544.Institute.
40. *SAS/STAT® 9.4 User's Guide*, 2nd ed.; SAS Institute Inc.: Cary, NC, USA, 2009.
41. Klem, T.B.; Bleken, E.; Morberg, H.; Thoresen, S.I.; Framstad, T. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. *Vet. Clin. Pathol.* **2010**, *39*, 221–226, doi: 10.1111/j.1939-165X.2009.00199.x.
42. Friendship, R.M.; Lumsden, J.H.; McMillan, I.; Wilson, M.R. Hematology and biochemistry reference values for Ontario swine. *Can. J. Comp. Med.* **1984**, *48*, 390, doi: 10.1111/j.1939-165X.2009.00199.x.
43. IZSLER, Parametri di Chimica Clinica: Valori Osservati in Suini di Diversa Età. Available online: https://www.izsler.it/pls/izs_bs/v3_s2ew_consultazione.mostra_pagina?id_pagina=1494 (accessed on 01 October 2020).
44. Mueller-Harvey, I. Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.* **2006**, *86*, 2010–2037, doi:10.1002/jsfa.2577.
45. Galassi, G.; Mason, F.; Rapetti, L.; Crovetto, G.; Spanghero, M. Digestibility and metabolic utilisation of diets containing chestnut tannins and their effects on growth and slaughter traits of heavy pigs. *Ital. J. Anim. Sci.* **2019**, *18*, 746–753, doi:10.1080/1828051X.2019.1570361.
46. Chapman, P.M. Defining hormesis: Comments on Calabrese and Baldwin. *Hum. Exp. Toxicol.* **2002**, *21*, 99–101, doi: 10.1191/0960327102ht218oa.
47. Bee, G.; Silacci, P.; Ampuero-Kragten, S.; Candek-Potokar, M.; Wealleans, A.; Litten-Brown, J.; Salminen, J.; Mueller-Harvey, I. Hydrolysable tannin-based diet rich in gallotannins has a minimal impact on pig performance but significantly reduces salivary and bulbourethral gland size. *Animal* **2017**, *11*, 1617–1625, doi:10.1017/S1751731116002597.

48. Williams, A.; Krych, L.; Ahmad, H.; Nejsum, P.; Skovgaard, K.; Nielsen, D.; Thamsborg, S. A polyphenol-enriched diet and *Ascaris suum* infection modulate mucosal immune responses and gut microbiota composition in pigs. *PLoS ONE* **2017**, *12*, e0186546, doi:10.1371/journal.pone.0186546.
49. Carstensen, L.; Ersbøll, A.K.; Jensen, K.H.; Nielsen, J.P. *Escherichia coli* post-weaning diarrhea occurrence in piglets with monitored exposure to creep feed. *Vet. Microbiol.* **2005**, *110*, 113–123, doi: 10.1016/j.vetmic.2005.07.011.
50. Laine, T.M.; Lyytikäinen, T.; Yliaho, M.; Anttila, M. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. *Acta Vet. Scand.* **2008**, *50*, 21, doi: 10.1186/1751-0147-50-21.
51. Etim, N.N.; Williams, M.E.; Akpabio, U.; Offiong, E.E.A. Haematological parameters and factors affecting their values. *Agric. Sci.* **2014**, *2*, 37–47, doi: 10.12735/as.v2i1p37.
52. Chedea, V.; Palade, L.; Pelmus, R.; Dragomir, C.; Taranu, I. Red grape pomace rich in polyphenols diet increases the antioxidant status in key organs kidneys, liver, and spleen of piglets. *Animals* **2019**, *9*, 149, doi:10.3390/ani9040149.
53. Abarghuei, M.J.; Rouzbehan, Y.; Salem, A.Z.M.; Zamiri, M.J. Nitrogen balance, blood metabolites and milk fatty acid composition of dairy cows fed pomegranate-peel extract. *Livest. Sci.* **2014**, *164*, 72–80, doi: 10.1016/j.livsci.2014.03.021.
54. Chen, G.J.; Song, S.D.; Wang, B.X.; Zhang, Z.F.; Peng, Z.L.; Guo, C.H.; Zhong, J.C.; Wang, Y. Effects of forage: Concentrate ratio on growth performance, ruminal fermentation and blood metabolites in housing-feeding yaks. *Asian Austral. J. Anim.* **2015**, *28*, 1736, doi: 10.5713/ajas.15.0419.
55. Hăbeanu, M.; Lefter, N.A.; Gheorghe, A.; Untea, A.; Ropotă, M.; Grigore, D.-M.; Varzaru, I.; Toma, S.M. Evaluation of performance, nitrogen metabolism and tissue composition in barrows fed an n-3 PUFA-rich diet. *Animals* **2019**, *9*, 234, doi: 10.3390/ani9050234.
56. Whang, K.; Easter, R. Blood urea nitrogen as an index of feed efficiency and lean growth potential in growing-finishing swine. *Asian- Asian Austral. J. Anim.* **2000**, *13*, 811–816, doi:10.5713/ajas.2000.811.
57. Bravo, L.; Abia, R.; Eastwood, M.A.; Saura-Calixtol, F. Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on coloic fermentation and faecal output. *Br. J. Nutr.* **1994**, *71*, 933–946, doi: 10.1079/BJN19940197.
58. Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317–333, doi: 0.1111/j.1753-4887.1998.tb01670.x.
59. Choy, Y.; Quifer-Rada, P.; Holstege, D.; Frese, S.; Calvert, C.; Mills, D.; Lamuela-Raventos, R.; Waterhouse, A. Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins. *Food Funct.* **2014**, *5*, 2298–2308, doi:10.1039/c4fo00325j.
    Choi, H.; Jeong, J.; Kim, D.; Lee, Y.; Kwon, H.; Kim, Y. Influence of rapeseed meal on growth performance, blood profiles, nutrient digestibility and economic benefit of growing-finishing pigs. *Asian Austral. J. Anim.* **2015**, *28*, 1345–1353, doi:10.5713/ajas.14.0802.

Experiment 3:

Evaluation of leonardite as a feed additive on lipid metabolism and growth of weaned piglets

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6.1. Abstract

We evaluated the effects of leonardite supplementation, mainly composed of humic acids (HAs), as a functional feed additive in weaned piglets. One hundred and twenty piglets (Large Withe × Landrace) were weaned at 28±2 days, and randomly divided into two groups (6 pens per group, 10 piglets per pen). After one week of adaptation, for 40 days groups were fed a control diet (CTRL) and an HA enriched diet (0.25% of leonardite; HAG). Body weight (BW), average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR) were measured throughout the experimental period. On the last day of the trial four piglets per pen were randomly selected and the blood was collected to evaluate the serum metabolic profile and diamine oxidase content. Chemical analyses showed that leonardite was characterized by a high content of ash 23.27% (as-fed basis), polyphenolic content of 35.18±3.91 mg TAEq/g, and an antioxidant capacity of 73.31±8.22 μmol TroloxEq/g. The HAG group showed an increase in BW, ADG and ADFI ($P < 0.01$) compared to the CTRL group during the experimental period. In terms of the serum metabolic profile, the HAG group showed a significant increase in total protein content ($P < 0.001$), albumin ($P < 0.001$), albumin/globulin ratio ($P < 0.01$), phosphatase alkaline ($P < 0.01$), calcium, phosphorus and magnesium ($P < 0.05$) compared to the CTRL group. A modulation in the serum lipid profile was recorded. The HAG group showed a decrease in total triglycerides ($P < 0.05$) with higher total cholesterol ($P < 0.05$), however only high-density lipoprotein showed a significant increase ($P < 0.001$) compared to the CTRL group. No significant differences in the amount of diamine oxidase were found between groups. In conclusion, leonardite inclusion in the diet at 0.25% was shown to have a positive effect on the serum lipid profile and animal growth. This thus suggests that leonardite can be considered as a new feed additive, which improves the health and performance of weaned piglets.

6.2. Introduction

Weaning is recognized a stressful period in intensively reared pigs, with a high occurrence of multifactorial diseases which are the most common reason for the use of antibiotics (Zhao et al., 2007). Managing weaning correctly is crucial, as it influences the use of antibiotics as well as long-term profitability. The European Food Safety Authority (EFSA) recommends adopting an integrated strategy in food-producing animals, reducing and also replacing the antibiotics with novel functional feed and additives (Cormican et al., 2017). Functional feed additives thus play a pivotal role, particularly concerning gastrointestinal disorders (Rossi et al., 2012; Heo et al., 2013).

Among many alternatives, leonardite which is used in veterinary practice for treating diarrhoea in horses, ruminants and poultry, has been proposed for preventing diarrhoea in animals (Ozturk et al., 2012; Domínguez-Negrete et al., 2019). Leonardite is a microbial-derived product mainly composed of humic acids (HAs), which are derived from the decomposition of organic matter, usually exploited for the fertilization of soil. HAs also protect the mucosa of the intestine, with recognized anti-inflammatory, antiphlogistic, adsorptive and antitoxic proprieties (Islam et al., 2005; Aksu and Bozkurt, 2009). Natural humic substances may provide benefits to piglets' health during post-weaning (Trckova et al., 2017). They have shown antioxidant proprieties that could sustain the animals during the stressful period of weaning. They have also shown antimicrobial activity against pathogens leading to a decreased incidence of diarrhoea and better growth performance also modulating the animal's metabolism (Wang et al., 2008; Aeschbacher et al., 2012). Humic acids and their sodium salts are permitted for oral use (inclusion level: 500-2000 mg/kg of body weight) in horses, ruminants, swine and poultry for the treatment of diarrhoea, dyspepsia and acute intoxications (EGTOP/1/2011). Lower levels of humic substances used as a feed additive (2-10 g/100g of diet) in the pigs' diet seem to demonstrate that they improve growth performance and meat quality, also reducing ammonia emissions from manure (Ji et al., 2006; Wang et al., 2008; Kim et al., 2019). Although encouraging findings concerning the use of HAs as a prophylactic tool for intestinal health have been reported, the inclusion of leonardite as a feed additive to promote growth, has not been extensively investigated. Several studies have revealed discordant findings *in vivo* probably due to the wide range of doses tested and the wide variability in the composition of humic-based products (Trckova et al., 2015; Kaevska et al., 2016; Trckova et al., 2018).

We focused on the inclusion of leonardite as a feed additive aimed at not substantially modifying the nutrient balance in the diet in order to simply be able to exploit the functional proprieties of leonartide. The aim of this study was thus to evaluate the effect of leonardite

included at 0.25%, as natural material rich in HAs, on the principal metabolic parameters and growth of weaned piglets. Leonardite was also assessed in terms of its chemical composition, phenolic content, and antioxidant capacity.

6.3. Materials and Methods

6.3.1. Chemical evaluation of experimental diets and leonardite

The experimental diets and leonardite (Commission Regulation EU 2017/1017), registration number 13.10.2; purchased from New Feed Team S.R.L. (Lodi, Italy) were characterized in terms of their principal constituents: humidity, ether extract (EE), crude protein (CP), crude fiber (CF), and ash contents. The samples were analysed in duplicate following official analysis methods (AOAC, 2005). Dry matter (DM) was obtained by inserting samples into previously weighed aluminium bags, which were dried in a forced-air oven at 105°C for 24 h (AOAC method 930.15).

Ash was obtained using a muffle furnace at 550°C (AOAC method 942.05). CP was determined by a Kjeldahl method (AOAC method 2001.11). EE was determined using ether extraction in the Soxtec system (DM 21/12/1998). CF was determined by the filtering bag technique (AOCS method Ba 6a-05).

The mineral composition of the two experimental diets and leonardite were evaluated after mineralization with inductively coupled plasma mass spectrometry (ICP-MS). First, calibration curves for each element considered (Na, Mg, Al, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Pb, P) were obtained using certified reference materials. Dried samples (0.3 g) were digested by a microwave digestion system (Anton Paar MULTIWAVE-ECO) in Teflon tubes filled with 10 mL of 65% HNO_3 by applying a one-step temperature ramp (at 120°C in 10 min and maintained for 10 min).

The mineralized samples were cooled for 20 minutes and then transferred into the polypropylene test tubes. Mineralized samples were diluted 1:100 with 0.3 M HNO_3 in MilliQ Water. The concentration of elements was measured by ICP-MS (BRUKER Aurora-M90 ICP-MS). In order to check the nebulization performance, an aliquot of a 2 mg/L of an internal standard solution (^{72}Ge, ^{89}Y, ^{159}Tb) was added to the samples to produce a final concentration of 20 µg/L. Polyatomic interference was removed using a collision-reaction interface (CRI) with an H_2 flow of 80 mL/min through skimmer cone.

The fatty acid profile of the experimental diets was analysed starting with the total lipid extraction and preparation of the fatty acid methyl esters (Christie and Han, 2003). The fatty acid analysis was carried out using gas chromatography (TRACE GC Ultra, Thermo Fisher

Scientific, Rodano, Italy) fitted with an automatic sampler (AI 1300, Thermo Fisher Scientific) and flame ionization detector (FID). An RT-2560 fused silica capillary column (100 m × 0.25 mm × 0.25 μm film thickness; Restek, Milan, Italy) was used with a programmed temperature from 80°C to 180 °C at 3°C/min, then from 180 °C to 250°C at 2.5 °C/min, which was then held for 10 min. The carrier gas was helium at 1.0 mL/min with an inlet pressure of 16.9 psi. A quantitative procedure was used where 1 mL of internal standard (1 mg/mL 23:0 methyl ester; N-23-M; Nu-Chek Prep Inc., Elysian, MN, USA) was added prior to methylation. The fatty acid methyl ester (FAME) contents were quantified by weight as a percentage of the total FAMEs. All analyses were performed in duplicate.

6.3.2. Polyphenolic content of leonardite

The phenolic content of leonardite was evaluated by the Folin-Ciocalteu method following Attard et al. (2013). Polyphenolic extract was obtained by diluting 2.5 g of sample with 15 mL of methanol (100%), which was then allowed to macerate for 24 h at room temperature. The samples were centrifugated (3000 rpm, 10 min) and the supernatants were collected and stored at -20° C for further polyphenol evaluation. The assay was performed by reacting 50 μL of extracted sample/standard with 500 μL of Folin-Ciocalteu reagent (10% in water) and 400 μL sodium carbonate (1 M). The reaction mixture was left to stand for 15 min in the dark and the total phenolic content was determined spectrophotometrically at 630 nm (JASCO V-630 UV-VIS, Germany). Calibration curves were prepared with tannic acid from 480 μg/mL to 15 μg/mL as standard. The results were expressed as mg/100g of tannic acid equivalent (mg TAEq/100g).

6.3.3. Trolox Equivalent Antioxidant Capacity assay (TEAC)

Leonardite was evaluated for its antioxidant proprieties using an ABTS radical cation discoloration assay (Prior et al., 2005). First the dried samples were diluted in water (100 mg/mL) and adjusted from an initial pH of 3.5 to pH 9 by adding NaOH (1M) thus facilitating the solubilization of humic acids. The sample was then stirred for 24 h at room temperature. The solution was centrifuged for 10 min at 3000 rpm, the supernatant was collected and stored at -20°C until the analysis. Before proceeding with the assay, the humic acid extract was adjusted to pH 7 with HCl (1M), filtered with an 0.45 μm syringe filter, and diluted in order to obtain a clear solution that would not alter the reading of the spectrophotometer. In order to assess a possible dose related antioxidant effect, the concentrations tested were: 5%, 2%, 1.25% of the humic acid original extract (Dell'Anno et al., 2020). The antioxidant activity was tested by adopting the ABTS assay, according to Prior et al. (2005).

The reaction mixture with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was generated by the reaction of 7 mM ABTS with 2.45 mM of K-persulfate. The reaction mixture was left to stand in the dark for 16 hours at room temperature and used within two days. The working solution of the $ABTS^{\bullet+}$ radical cation was obtained by diluting $ABTS^{\bullet+}$ in ethanol in order to obtain an absorbance of 0.700 ± 0.02 OD at 734 nm at room temperature. First, a calibration curve was obtained using different concentrations (2000 μM, 1500 μM, 1000 μM, 500 μM, 100 μM, 0 μM) of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as standard. The assay was performed using 10 μL of diluted sample (standard and extract) added to 1 mL of working solution ($ABTS^{\bullet+}$). The absorbances were recorded spectrophotometrically (JASCO V-630 UV-VIS, Germany) at 734 nm from 1 to 6 minutes.

The total antioxidant capacity was expressed as the percentage inhibition (PI), according to the equation: PI = [($AbsABTS^{\bullet+}$ - Abs sample) /$AbsABTS^{\bullet+}$] × 100, where $AbsABTS^{\bullet+}$ denotes the initial absorbance of diluted $ABTS^{\bullet+}$, and the Abs sample denotes the absorbance of the sample at every 6 mins of reaction. Using appropriate calibration curves, the result was expressed as the equivalent concentration of μmol Trolox/g after six minutes of reaction (μmol TroloxEq/g).

6.3.4. Experimental design and sample collection

The experimental trial was approved by the Animal Welfare Body of the University of Milan (protocol No. 31/2019) and performed in accordance with European regulations (Commission Directive 2010/6/EU). It was conducted on a commercial farm that was free from pathologies included in the ex-list A of the World Organization for Animal Health (Porcine Reproductive Respiratory Syndrome, atrophic rhinitis, transmissible gastroenteritis, salmonellosis, Aujeszky disease).

A total of 120 crossbred piglets (Large White × Landrace), weaned at 28±2 days (50% female and 50% male), were housed in 12 different pens, in homogeneous environmental conditions (27°C and 60% relative humidity). In order to guarantee a homogeneous weight intra-pen and inter-group, the piglets (10 animals/pen) were randomly assigned to the control group (CTRL: 6 pens, 60 piglets) and treatment with an HA enriched diet (0.25% of leonardite; HAG: 6 pens, 60 piglets).

The two experimental isoproteic and isoenergetic diets (Table 21) were formulated using Plurimix software (Fabermatica, Cremona, Italy) in order to meet the nutritional requirements for post-weaned piglets (NRC, 2012) and were provided by Ferraroni S.p.a. (Cremona, Italy). Both experimental diets were formulated including 1% coconut oil (Table 21), a raw ingredient

characterized by high digestibility, as an enhancer of palatability for young animals, taking into account the balance in the saturated and unsaturated fatty acid ratio.

Table 21. Diet composition of *in vivo* trial (% as fed basis) divided by control (CTRL) and treatment group fed with HA enriched diet (0.25% leonardite).

Items	CTRL	HAG
Ingredients, % as fed basis		
Barley, meal	25.15	25.15
Wheat, meal	19.41	19.36
Corn, flakes	14.03	13.83
Corn, meal	4.85	4.85
Soybean, meal	4.65	4.65
Soy protein concentrates	4.11	4.11
Biscuits, meal	4.00	4.00
Dextrose monohydrate	3.50	3.50
Wheat middling	4.32	4.32
Whey protein concentrate	3.00	3.00
Fish, meal	2.50	2.50
Milk whey	2.50	2.50
Coconut oil	1.00	1.00
Soy oil	1.00	1.00
Plasma, meal	1.00	1.00
Organic Acids[1]	1.00	1.00
Dicalcium phosphate	0.85	0.85
Animal fats	0.70	0.70
L-Lysine	0.50	0.50
Benzoic acid	0.40	0.40
L-Threonine	0.35	0.35
DL-Methionine	0.35	0.35
Sodium Chloride	0.27	0.27
Vitamins[2]	0.25	0.25
L-Valine (96.5%)	0.15	0.15
L-Tryptophan	0.08	0.08
Flavouring[3]	0.04	0.04
Copper sulphate	0.04	0.04
Leonardite	-	0.25
Calculated nutrient levels[4], % as fed basis		
Crude protein	16.92	16.88
Ether extract	5.06	5.19
Crude fiber	3.15	3.22
Ashes	5.1	5.1
DE[5] (Mc/Kg)	3.43	3.43

[1]Organic Acids: formic acid, sodium formate, sorbic acid, orthophosphoric acid, calcium formate, citric acid, and fumaric acid.
[2]Vitamins and vitamin-like compounds per kg: Vitamin A, 10,000; Vitamin D3, 1,000 IU; Vitamin E, 100 mg; Vitamin B1, 3 mg; Vitamin B2, 96.3 mg; Vitamin B6, 5.8 mg; Calcium D-pantothenate, 27 mg; Vitamin B12, 0.040 mg; Vitamin K3, 4.8 mg; Biotin, 0.19 mg; Niacinamide, 35 mg; Folic Acid, 1.4 mg. Choline chloride 120 mg, Betaine chloride 70 mg.
[3]Vanilla flavouring.
[4]Calculation performed with Purimix software (Fabermatica, Cremona, Italy).
[5]DE: digestible energy content estimated from NRC (2012).
HA: humic acids; HAG: humic acid enriched diet group supplemented with 0.25% leonardite.

After an adaptation period of seven days with the same basal diet, piglets were fed the experimental diets (CTRL and HAG) *ad libitum* from day 0 to day 40. Body weight (BW) was individually recorded using a validated scale at the beginning (d 0), day 14 (d 14), day 28 (d 28) and day 40 (d 40). Feed intake was recorded weekly for each pen by measuring the feed refuse per pen. Based on the BW results, the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated. In terms of zootechnical performance, each pen was considered as the experimental unit. At day 40, four animals from each pen were randomly selected, in order to collect faeces from the rectal ampulla of four animals per pen (total samples CTRL: n=24; total samples: HAG n=24), and blood samples from the jugular vein of four animals per pen (total samples CTRL: n=24; total samples: HAG n=24) using vacuum tubes. The health status was evaluated daily and individual faecal consistency was scored weekly using a four-level scale: 0 = normal (faeces firm and well formed), 1 = soft consistency (faeces soft and formed), 2 = mild diarrhoea (loose faeces, usually yellowish), 3 = severe diarrhoea (faeces watery and projectile). A faecal consistency score ≤ 1 (0, 1) was considered normal, whereas a faecal score > 1 (2, 3) was defined as indicative of diarrhoea (Rossi et al., 2014).

6.3.5. Biological sample analysis

Blood samples (4 animals/pen) were allowed to clot for 2 hours at room temperature. Serum samples were obtained by centrifugation and were evaluated by a multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA) at 37 °C for the following metabolic parameters: total protein content, albumin, globulin, albumin/globulin (A/G), urea, alanine aminotransferase (ALT-GPT), aspartate aminotransferase (AST-GOT), phosphatase alkaline (ALP), total bilirubin, glucose, total cholesterol, calcium (Ca), phosphorus (P), magnesium (Mg), total triglycerides, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and creatinine.

Diamine oxidase concentrations in serum samples (dilution 1:10), as an indirect marker of intestinal integrity (Zhang et al., 2013), were evaluated using a DAO enzyme-linked immunosorbent assay (DAO; Wuhan Fine Biotech Co., Ltd., China). The following conditions were used: 96-well pre-coated microplates were washed twice, after which 100 µL of standard or sample were added to each well and incubated at 37°C for 90 min. The plates were washed and 100 µL of diluted detection antibody were added to each well and incubated at 37°C for 60 min. After washing, 90 µL of TMB substrate solution were added to each well. The plate was left in the dark at 37°C for 15 min, then the reaction was stopped by adding 50 µL of stop solution to each well. Absorbance was measured on a plate reader at 450 nm (Bio-Rad 680

Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, California, USA). For each plate, a calibration curve was adopted to calculate the DAO concentration of each sample using Curve Expert v. 1.4 software. The concentrations determined were expressed as nanograms of DAO per mL (ng/mL).

Collected fecal samples were analysed by RT-PCR to estimate the bacterial DNA abundance of the main enteric microorganisms as the parameter of gut health. Bacterial DNA was extracted as reported by Patrone et al. (2018). Copy numbers of the 16S rRNA gene from *Escherichia coli*, Enterobacteriaceae, *Bifidobacterium* spp. and *Lactobacillus* spp. were quantified using previously reported primers (Penders et al. 2005, Bartosch et al. 2004, Byun et al., 2004). Quantification was carried out in triplicate using the LightCycler 480 Instrument II (Roche Diagnostics, Monza, Italy). *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae were quantified using the KAPA SYBRR FAST (Kapa Biosystems, Inc; Wilmington, MA) containing a 300 nM final primer concentration. On the other hand, *E. coli* was quantified using the KAPA Probe FAST Master mix (Kapa Biosystems, Inc; Wilmington, MA) containing 500nM of primers and 100 nM of the probe (final concentration). The primers and probes used for the quantification of *E. coli* were described by Penders et al (2005). *Bifidobacterium infantis* ATCC 15697D and *E. coli* ATCC 700926D-5 genomic DNAs, used for preparing standard curves, were provided by the American Type Culture Collection (ATCC). Genomic DNA of *Lactobacillus fermentum* DSM20052 was obtained by extracting 5 mL of activated culture using the Genomic DNA extraction Kit (Promega) and quantified with a Qubit™ fluorometer (Invitrogen, Milan, Italy). Standard curves were obtained by 10-fold dilutions of genomic DNA for each reference genomic DNA. Results were expressed as ng of target DNA/ng of total fecal bacterial DNA. The four samples analysed for each pen were considered as replicates for each treatment.

6.3.6. Statistical analysis

Data on zootechnical performance, biological samples, antioxidant capacity and diamine oxidase were evaluated using SAS 9.4 (SAS Inst. Inc., Cary, NC). Before analyses, all data were tested for normality with the Shapiro-Wilk test (for values > 0.9 data were considered normally distributed). The data were analysed with the general linear model. The model included the effect of treatments, and for the zootechnical performance, the effect of time (Day) and the interaction between treatment and time (TRT x Day) were included. Tukey-Kramer studentized adjustments were used to separate the means, and the results were reported as LSMEANS and SEM or standard error (SE). Means were considered different when $P \leq 0.05$ and tended to different if $0.05 < P \leq 0.1$.

6.4. Results and Discussion

6.4.1. Chemical characterization of experimental diets and leonardite

Leonardite is a heterogeneous mixture of polydisperse material formed by humification. The variability of the environment during humification significantly influences the final composition of leonardite. This is confirmed by the wide heterogeneity of commercial products for animal nutrition. The chemical characterization of leonardite enabled our results to be compared with other findings.

Our results revealed that the main component of leonardite was ash, corresponding to 23.27%, confirming the high content of minerals although a wide range form 15 to 70% on dry basis is reported in the literature (Chammui et al., 2014). Our experimental diets showed a mineral content in line with regulations (Regulation EC 1831/2003). Nevertheless, the high level of minerals did not notably alter the mineral levels as a result of the inclusion of 0.25% of leonardite in the diet, considering the maximum levels permitted by Reg. EU 1831/2011. This percentage inclusion also reduced the dustiness and obtained optimal mixing conditions during the feed production phases. The inclusion of leonardite did not affect the fatty acid profile of the diets (Table 22).

Table 22. Chemical composition of diets for control group (CTRL), treatment group (HAG) and leonardite (LEO). All values are expressed as percentage as fed-basis (%).

	CTRL	**HAG**	**LEO**
DM	91.62	91.62	81.13
CP	16.24	16.57	6.15
EE	3.79	3.87	0.6
CF	2.69	2.24	5.15
Ashes	4.59	4.46	23.27

FA composition (% total FAMEs)		
	CTRL	**HAG**
C 8:0	0.78	0.80
C 10:0	0.74	0.81
C 12:0	6.68	7.26
C 14:0	3.82	4.05
C 14:1	0.01	0.02
C 15:0	0.09	0.09
C 16:0	16.36	16.32
C 16:1	0.81	0.81
C 17:0	0.13	0.12
C 17:1	0.06	0.06
C 18:0	4.87	4.85
C 18:1 n9 trans	0.06	0.06
C 18:1 n9 cis	24.15	23.71

C 18:2 n6 cis	35.49	35.18
C 20:0	0.29	0.28
C 18:3 n6	0.04	0.02
C 20:1	0.67	0.63
C 18:3 n3	2.62	2.64
C 21:0	0.02	0.03
C 20:2	0.10	0.14
C 22:0	0.19	0.19
C 20:3 n6	0.01	0.01
C 22:1 n9	0.04	0.04
C 20:3 n3	0.04	0.03
C 20:4 n6	0.11	0.09
C 22:2	0.02	0.02
C 24:0	0.15	0.14
C 20:5 n3	0.64	0.65
C 24:1	0.05	0.04
C 22:6 n3	0.90	0.92

DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fiber; FA: fatty acids; FAMEs: fatty acid methyl esters; CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% of leonardite; LEO: leonardite, humic acid-based feed ingredient.

Leonardite showed a high content of Ca, Fe and Al. The contaminants (As, Pb, Cd) were scarce and below the safety limits (Commission Regulation EU 1275/2013). Moreover, no contaminants (under the detection limits) were revealed in the diet supplemented with 0.25% leonardite. In order to reduce the emission of heavy metals into the environment and optimize the diet for a better exploitation of macro and microelements, the level of minerals in feed ingredients should always be evaluated in order to guarantee the correct diet formulation (Hejna et al., 2019).

6.4.2. Polyphenolic content of leonardite

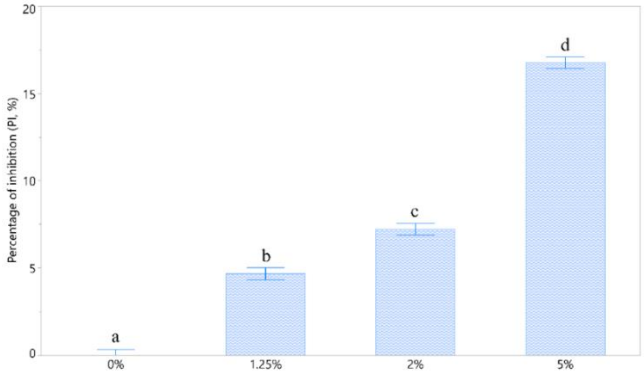
Results form the Folin-Ciocalteu method revealed that leonardite was characterized by 35.18±3.91 mg TAEq/100g of polyphenols. Polyphenols such as hydroxytyrosol from olive oil, isoflavones from soy proanthocyanidins in grape seed extracts (Mennen et al., 2005), were evaluated as indirect indicators of the antioxidant proprieties of humic substances. The recommended dose of polyphenol intake in the human diet is 396 mg/d (Ma and Chen, 2020). Polyphenols are commonly found in plants as secondary metabolites, which aid the plant in structural development and react to many biotic and abiotic stressors with well recognized antioxidant activities (Jessica et al., 2019). Our results confirmed that humic acids are a source of antioxidant compounds (Karadirek et al., 2016; Lv et al., 2018). Considering the variability in the phenolic content of microbial-originated products, our results enabled us to identify the properties leonardite.

Although the amount of polyphenols obtained was lower compared with other feed and food products (Castrica et al., 2019), the leonardite antioxidant capacity should be considered high because the phenolic content is comparable to round melon (*Praecitrullus vulgaris*). However, its antioxidant capacity is higher than the µmol TroloxEq/g content of lemon and orange water-soluble extracts (Kaur and Kapoor, 2002; Nilsson et al., 2005) which are considered as beneficial foods with positive effects on health. Humic acids can thus be considered as promising biologically-active natural antioxidants for the development of new classes of pharmaceuticals for medicine (Khil'ko et al., 2011).

6.4.3. Trolox Equivalent Antioxidant Capacity (TEAC) of leonardite

Our results showed that the humic acid extract contained a dose-dependent antioxidant capacity (Figure 16; $P < 0.0001$), with a measured amount of 73.31±8.22 µmol TroloxEq/g after 6 minutes. This is in line with other studies on the antioxidant activity of humic substances (Aeschbacher et al., 2012; Smirnova et al., 2012), thus suggesting that the effect is probably related to the content of phenolic moieties as the most important electron-donating groups. In addition, the presence of acid groups (-COOH, -OH) suggests that these substances are capable of an antioxidant effect (Smirnova et al., 2012). The effectiveness of humic substances related to their dose-effect response has also been observed by the gas-volumetric method (Efimova et al., 2012). The results obtained from the latter study showed that leonardite has an antioxidant capacity, highlighting its possible positive effects on animal health by reducing oxidation.

Figure 16. Percentage inhibition of $ABTS^{\bullet+}$ of different concentrations of humic acids extract (blank: 0%; 1.25%; 2% and 5%) measured by Trolox Equivalent Antioxidant Capacity (TEAC) assay.



[a-b] means with different superscripts are significantly different between treatments ($P < 0.0001$). Data are expressed as least square means (LSMEANS) and Standard Error (SE).
ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

6.4.4. Zootechnical performance

The final average body weight increased significantly in HAG piglets compared to the CTRL group confirming the positive effect of leonardite treatment on animal growth. The HAG group showed a higher average BW at d 40 compared to CTRL ($P < 0.001$). HAG also showed a higher ADG from d 14-28 and from d 28-40 ($P < 0.001$, Table 23). The ADFI of the HAG group increased from d 14-28 and d 28-40 ($P < 0.01$; Table 23) compared to the CTRL group. The higher final body weight of the HAG group was due to a higher consumption of feed although the feed conversion rate did not show significant differences between groups ($P > 0.05$).

Table 23. Zootechnical performance of *in vivo* trial (from day 0 to 40) divided by control (CTRL) and treatment (HAG supplemented with 0.25% of leonardite) group.

				P-values		
	CTRL	**HAG**	**SEM±**	Trt	Day	Trt x Day
BW, kg				0.112	< 0.001	< 0.001
d 0	8.71	8.72	0.871			
d 14	11.11	12.21				
d 28	15.44	18.36				
d 40	20.17[a]	24.25[b]				
ADFI, kg/d				0.003	< 0.001	0.254
d 0-14	0.353	0.465	0.034			
d 14-28	0.651[a]	0.841[b]				
d 28-40	0.730[a]	0.891[b]				
ADG, kg/d				< 0.001	< 0.001	0.535
d 0-14	0.171	0.249	0.024			
d 14-28	0.310[a]	0.440[b]				
d 28-40	0.396[a]	0.491[b]				
FCR, kg/kg						
d 0-14	1.97	1.77	0.115	0.384	0.146	0.445
d 14-28	1.93	1.79				
d 28-40	2.03	2.09				

[a-b] means with different superscripts are significantly different between treatments ($P < 0.05$).
Data are expressed as least squares means (LSMEANS) and standard error of the mean (SEM).
BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FRC: feed conversion rate; CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% of leonardite.

Wang et al. (2008) observed a raised ADG with the inclusion of humic substances at 5% and 10% in the diets of the pigs. Trckova et al. (2018) also demonstrated the positive effect of leonardite (supplementation levels of 20 g/kg in the diet) on BW, ADFI and ADG parameters of weaned piglets. Although it is still not clearly exactly how leonardite exerts its action, the improvements in zootechnical performance seem to be related to the capacity of humic substances to help ion transport through membranes, the protection of intestinal mucosa, the

enhancement of enzymes activities, and the better nutrient digestion and adsorption (particularly proteins and minerals) (Trckova et al., 2018). Bai et al. (2013) estimated that the optimum level for increasing zootechnical performance was a 0.25% supplementation of fulvic acid. Therefore, a low concentration of HA inclusion may improve the growth performance with no influence on the diet composition and feed preparation.

Regarding the health status of the animals throughout the experimental period, only one piglet died at d 7 in the CTRL group. This amounts to a mortality incidence of < 1%, which could be considered as normal in common livestock farming. In both groups, clinical signs of diarrhoea occurred from d 14 to d 21 and was transient (on average lasting three days). The occurrence of diarrhoea (number of new animals with fecal score > 1/total animals per group) was 18.6% and 16.7% in the CTRL and HAG groups at d 14, respectively. The occurrence of diarrhoea was below the average post weaning levels (Carstensen et al., 2005; Laine et al., 2008). Our results confirmed the increase in BW, ADFI and ADG with a leonardite inclusion of 0.25%, demonstrating that a lower inclusion level could equally enhance the zootechnical performance thus also optimizing the use of leonardite as a feed additive.

6.4.5. Biological sample analysis

The results of the serum metabolic parameters showed that the HAG group had higher levels of total proteins, albumin, A/G ratio, phosphatase alkaline, glucose, cholesterol, calcium, phosphorous, magnesium, high density lipoprotein, creatinine and total triglycerides ($P < 0.05$, Table 24).

Table 24. Metabolic profile analysis of blood serum divided by control (CTRL) and treatment group (HAG supplemented with 0.25% of HA) measured at day 40.

Analyte	CTRL	HAG	SEM±	P-Value
Total protein content, g/L	52.88[a]	61.45[b]	1.33	<0.001
Albumin, g/L	19.31[a]	26.90[b]	1.25	<0.001
Globulin, g/L	33.58	34.57	1.10	0.535
Albumin/Globulin (A/G)	0.58[a]	0.81[b]	0.05	0.002
Urea, mmol/L	2.18	2.30	0.32	0.799
Alanine aminotransferase (ALT-GPT), IU/L	38.33	47.80	3.79	0.093
Aspartate aminotransferase (AST-GOT), IU/L	54.17	50.10	3.32	0.397
Phosphatase alkaline (ALP), IU/L	165.67[a]	228.50[b]	14.41	0.006
Total bilirubin, µmol/L	1.98	2.40	0.24	0.238
Glucose, mmol/L	5.00	5.94	0.35	0.075
Calcium, mmol/L	2.28[a]	2.65[b]	0.06	<0.001
Phosphorus, mmol/L	3.05[a]	3.73[b]	0.09	<0.001
Magnesium, mmol/L	0.85[a]	0.97[b]	0.03	0.014
Creatinine, µmol/L	78.92	90.80	4.43	0.073

Total cholesterol, mmol/L	2.51[a]	2.92[b]	0.12	0.024
High density lipoprotein (HDL), mmol/L	0.77[a]	1.00[b]	0.03	<0.001
Low density lipoprotein (LDL), mmol/L	1.60	1.82	0.09	0.099
Triglycerides, mmol/L	0.68[a]	0.51[b]	0.05	0.027

All values were in the physiological range confirming that leonardite did not negatively affect the health status and should be considered as a safe feed additive. An increased albumin and total protein content are related to the age of piglets and their rapid growth (de Meer et al., 2000). The increased A/G ratio was a consequence of the higher albumin value, however no significant difference was observed for the globulin content, whose increase is often related to an inflammatory process (Bertoni et al., 2008).

The ALP content in serum showed a significant difference between the CTRL and HAG ($P < 0.05$) groups. ALP is an important marker of bone remodelling which is involved in cartilage maturation and calcification. ALP in serum is mainly synthetized by liver and bones, and is involved in the formation of phosphorous ions, whose combination with calcium leads to the formation of bone salts (Yuan et al., 2011). The increased ALP level in the HAG group could be explained by a growth burst of the piglets, also connected with a higher level of calcium and phosphorous in serum ($P < 0.05$).

The higher amount of serum magnesium ($P < 0.05$) in the HAG group suggests an enhanced response to stressors. Mg plays a crucial role as an enzymatic cofactor. When the animal is subjected to high levels of stress, catecholamines and stress-associated hormones are released leading to a shift in Mg from the intracellular to the extracellular space. This mechanism increases the urinary excretion of magnesium and subsequent decreases in the serum Mg concentrations. Thus, low serum Mg concentrations increase the release of stress-related hormones, thus establishing a feedback mechanism (Pouteau et al., 2018). Trckova et al. (2018) observed an increased amount of serum Mg, Ca and P in weaned piglets supplemented with leonardite (20 g/kg of diet), thus confirming our findings and suggesting that humic substances could influence the mineral content of serum.

Although the fatty acid profile of the experimental diet was not affected by the inclusion of leonardite, the HAG group exhibited an increased concentration of total cholesterol and decreased triglyceride levels compared with the CTRL group ($P < 0.05$). The increased cholesterol levels could be related to an increased HDL ($P > 0.05$) without any changes in the LDL amount. The increase in HDL may be related to an increased Mg concentration, whose presence or absence is strictly related to the incidence of cardiovascular and metabolic diseases (Verma and Garg, 2017). These results may also be due to the presence of phenolic compounds

in leonardite which are recognized by EFSA as important nutrients in protecting blood lipids from oxidative damage and the maintenance of normal blood HDL-cholesterol concentrations without increasing LDL (EFSA, 2011; 2012).

In fact, total cholesterol concentration is an index of the lipometabolic status which includes the free and bounded forms of HDL (Wang et al., 2011). HDL protects blood vessels by decreasing cholesterol levels in the blood stream and guaranteeing their stabilization (Grela and Klebaniuk, 2007). These results suggest that leonardite has a positive effect on the lipidic metabolism as well as protecting blood vessels.

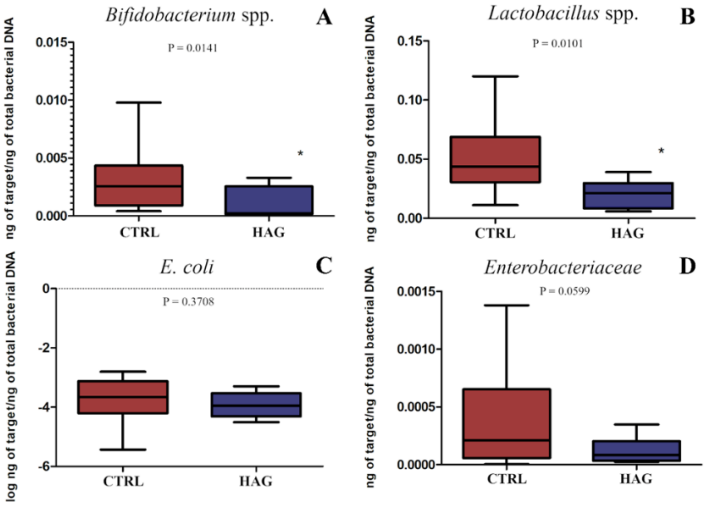
Results from the serum DAO analysis did not show any significant differences between the CTRL (289.40±48.30 ng/mL) and HAG (263.13±33.95 ng/mL) groups. The intact intestinal barrier plays a central role in preventing systemic infection and in the maintenance of the health status. DAO is abundantly expressed in the duodenal and jejunal mucosa and, therefore, DAO activity is a non-invasive marker of alterations in intestinal mucosal function and structure. Our results are in line with Liu et al. (2016) who showed an improvement in the gut barrier function, decreasing enteric diseases even without significant changes in serum DAO. An increased DAO level is often related to damage of the gut epithelium which releases this enzyme into the blood stream. Thus, the presence of DAO in healthy animals is usually scarce (Hou et al., 2014). Considering no occurrence of diarrhoea during the biological sample collection, although not representing the only indicator of a gut's barrier function, the detected levels of DAO did not reveal any intestinal alteration in either of the experimental groups.

Our RT-PCR data on fecal samples showed a significant reduction in bifidobacteria and lactobacilli in the HAG compared to the CTRL groups. Regarding *E. coli* and Enterobacteriaceae, no difference in abundance was observed.

Based on these preliminary data, it seems that leonardite particularly affects some groups of bacteria. Diarrhoea in piglets is often associated with specific pathogens, however the alteration in the gut microbiota composition can also be involved. In fact, Yang et al. (2017) analysed the gut microbiota of diarrheic neonatal piglets in which no pathogenic *E. coli* were detected. The authors found an increase in *Prevotella* spp. and a reduction in *E. coli* and some beneficial bacteria belonging to the Firmicutes phylum. This altered microbiota led to diarrhoea in neonatal piglets. Yang et al. (2017) results indicate that changes in terms of the relationship between different groups of bacteria can provoke the development of piglet diarrhoea. These microbiota modifications cause the onset of the inflammatory state as well as different nutrient degradation and adsorption abilities. Further analyses are required to investigate the ability of

leonardite to modulate the composition of gut microbiota in order to understand the mechanisms of its beneficial effects on piglet performance that we found in our study (Figure 17).

Figure 17. Target DNA of principal microbial indicators of gut microbiota (*A: Bifidobacterium spp., B: Lactobacillus spp., C: Escherichia coli, D: Enterobacteriaceae*) on total bacterial DNA analysed with RT-PCR divided by control and LEO group supplemented with 0.25% leonardite.



*means statistical differences between groups ($P < 0.05$)
CTRL: control group; HAG: humic acid enriched diet group supplemented with 0.25% leonardite.

6.5. Conclusions

Dietary supplementation with 0.25% leonardite improved the zootechnical performance, serum lipidic profile and gut epithelium integrity, thus indicating a good general health status. The increased serum HDL and decreased total triglycerides suggest that leonardite is a promising feed additive to improve lipid metabolism. The higher serum Mg content found also suggests that leonardite supports an improved stress response in weaned piglets.

6.6. References

Aeschbacher, M., Graf, C., Schwarzenbach, R., Sander, M., 2012. Antioxidant Properties of Humic Substances. Environmental Science & Technology 46, 4916-4925.

Aksu, T., Bozkurt, A., 2009. Effect of Dietary Essential Oils and/or Humic Acids on Broiler Performance, Microbial Population of Intestinal Content and Antibody Titres in the Summer Season. Kafkas Universitesi Veteriner Fakultesi Dergisi 15, 185-190.

AOAC, 2005. Official Methods of Analysis, Washington, DC.

Attard, E., 2013. A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. Central European Journal of Biology 8, 48-53.

Bai, H.X., Chang, Q.F., Shi, B.M., Shan, A.S., 2013. Effects of fulvic acid on growth performance and meat quality in growing-finishing pigs. Livestock science 158(1-3), 118-123.

Bartosch, S., Fite, A., Macfarlane, G.T., McMurdo, M.E., 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 70, 3575-3581.

Bertoni, G., Trevisi, E., Han, X., Bionaz, M., 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of Dairy Science 91, 3300-3310.

Byun R., Nadkarni M.A., Chhour K.L., Martin F.E., Jacques N.A., Hunter N., 2004. Quantitative analysis of diverse Lactobacillus species present in advanced dental caries. J Clin Microbiol 42, 3128-3136.

Carstensen, L., Ersbøll, A.K., Jensen, K.H., Nielsen, J.P., 2005. *Escherichia coli* post-weaning diarrhoea occurrence in piglets with monitored exposure to creep feed. Veterinary microbiology 110(1-2), 113-123.

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

Chammui, Y., Sooksamiti, P., Naksata, W., Thiansem, S., Arqueropanyo, O., 2014. Removal of arsenic from aqueous solution by adsorption on Leonardite. Chemical Engineering Journal 240, 202-210.

Christie, W.W., Han, X., 2003. Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids. Third edition, The Oily Press, Bridgwater, England.

Commission Directive 2010/6/EU, amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards mercury, free gossypol, nitrites and Mowrah, Bassia, Madhuca (Text with EEA relevance).

Commission Regulation EU 1275/2013, of 6 December 2013 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic, cadmium, lead, nitrites, volatile mustard oil and harmful botanical impurities (Text with EEA relevance).

Commission Regulation EU 2017/1017, Catalogue of feed materials (Text with EEA relevance.).

Cormican, M., Hopkins, S., Jarlier, V., Reilly, J., Simonsen, G., Strauss, R., et al., 2017. ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. Efsa Journal 15.

de Meer, K., Smolders, H., Meesterburrie, J., de Sain-van der Velden, M., Voorbij, H., Okken, A., Reijngoud, D., Kulik, W., 2000. A single food bolus stimulates albumin synthesis in growing piglets. Journal of Pediatric Gastroenterology and Nutrition 31, 251-257.

Dell'Anno, M., Sotira, S., Rebucci, R., Reggi, S., Castiglioni, B., Rossi, L., 2020. *In vitro* evaluation of antimicrobial and antioxidant activities of algal extracts. Italian Journal of Animal Science 19(1), 103-113.

Domínguez-Negrete, A., Gómez-Rosales, S., Angeles, M. D. L., López-Hernández, L. H., Reis-de Souza, T. C., López-García, Y., Zavala-Franco, A., Téllez-Isaias, G., 2019. Effect of the Addition of Humic Substances as Growth Promoter in Broiler Chickens Under Two Feeding Regimens. Animals 9(12), 1101, 1-15.

Efimova, I., Khil'ko, S., Smirnova, O., 2012. Antioxidant Activity of Humic Acids in Radical-Chain Oxidation Processes. Russian Journal of Applied Chemistry 85, 1351-1354.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), maintenance of normal blood pressure (anti-inflammatory properties"(ID 1882),"contributes to the upper respiratory tract health"(ID 3468),"can help to maintain a normal function of gastrointestinal tract"(3779), and "contributes to body defences against external agents" (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal, 9(4), 2033.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012. Scientific Opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006. EFSA Journal, 10(8), 2848.

EGTOP/1/2011, Expert Group for Technical Advice on Organic Production. Final Report on Feed.

Grela, E.R., Klebaniuk, R., 2007. Chemical composition of garlic preparation and its utilization in piglet diets. Medycyna Weterynaryjna 63, 792-795.

Hejna, M., Moscatelli, A., Onelli, E., Baldi, A., Pilu, S., Rossi, L., 2019. Evaluation of concentration of heavy metals in animal rearing system. Italian Journal of Animal Science 18, 1372-1384.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J., Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. Animal Physiology and Animal Nutrition 2, 207-237.

Hou, Y., Wang, L., Yi, D., Ding, B., Chen, X., Wang, Q., Zhu, H., Liu, Y., Yin, Y., Gong, J., Wu, G., 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. British Journal of Nutrition 111, 1748-1758.

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

Jessica, P., Amadou, D., Louis-Charles, R., Simon, B., 2019. Bioextracting Polyphenols from the Brown Seaweed Ascophyllum nodosum from Québec's North Shore Coastline. Industrial Biotechnology 15, 212-218.

Ji, F., McGlone, J., Kim, S., 2006. Effects of dietary humic substances on pig growth performance, carcass characteristics, and ammonia emission. Journal of Animal Science 84, 2482-2490.

Kaevska, M., Lorencova, A., Videnska, P., Sedlar, K., Provaznik, I., Trckova, M., 2016. Effect of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on faecal microbiota composition in weaned piglets. Veterinarni Medicina 61, 328-336.

Karadirek, Ş., Kanmaz, N., Balta, Z., Demirçivi, P., Üzer, A., Hızal, J., Apak, R., 2016. Determination of total antioxidant capacity of humic acids using CUPRAC, Folin–

Ciocalteu, noble metal nanoparticle- and solid–liquid extraction-based methods. Talanta 153, 120-129.

Kaur, C., Kapoor, H.C., 2002. Anti-oxidant activity and total phenolic content of some Asian vegetables. International Journal of Food Science & Technology 37(2), 153-161.

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

Kim, K., Bae, I., Cho, J., Choi, Y., Ha, J., Choi, J., 2019. Effects of Humic Acid and Blueberry Leaf Powder Supplementation in Feeds on the Productivity, Blood and Meat Quality of Finishing Pigs. Food Science of Animal Resources 39, 276-285.

Laine, T.M., Lyytikäinen, T., Yliaho, M., Anttila, M., 2008. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. Acta Veterinaria Scandinavica 50(21), 1-11.

Liu, G., Guan, G., Fang, J., Martinez, Y., Chen, S., Bin, P., Duraipandiyan, V., Gong, T., Tossou, M., Al-Dhabi, N., Yin, Y., 2016. Macleaya cordata Extract Decreased Diarrhea Score and Enhanced Intestinal Barrier Function in Growing Piglets. Biomed Research International 1-7.

Lv, J., Han, R., Huang, Z., Luo, L., Cao, D., Zhang, S., 2018. Relationship between Molecular Components and Reducing Capacities of Humic Substances. Acs Earth and Space Chemistry 2, 330-339.

Ma, G., Chen, Y., 2020. Polyphenol supplementation benefits human health via gut microbiota: A systematic review via meta-analysis. Journal of Functional Foods 66, 103829, 1-11.

Mennen, L.I., Walker, R., Bennetau-Pelissero, C., Scalbert, A., 2005. Risks and safety of polyphenol consumption. The American Journal of Clinical Nutrition 81(1), 326S–329S.

Nilsson, J., Pillai, D., Önning, G., Persson, C., Nilsson, Å., Åkesson, B., 2005. Comparison of the 2, 2′-azinobis-3-ethylbenzotiazo-line-6-sulfonic acid (ABTS) and ferric reducing anti-oxidant power (FRAP) methods to asses the total antioxidant capacity in extracts of fruit and vegetables. Molecular nutrition & food research 49(3), 239-246.

NRC, 2012. Nutrient Requirements of Swine. National Research Council, The National Academies Press, Washington, DC.

Ozturk, E., Ocak, N., Turan, A., Erener, G., Altop, A., Cankaya, S., 2012. Performance, carcass, gastrointestinal tract and meat quality traits, and selected blood parameters of broilers fed diets supplemented with humic substances. Journal of the Science of Food and Agriculture 92(1), 59-65.

Patrone, V., Minuti, A., Lizier, M., Miragoli, F., Lucchini, F., Trevisi, E., Rossi, F., Callegari, M.L., 2018. Differential effects of coconut versus soy oil on gut microbiota composition and predicted metabolic function in adult mice. BMC Genomics 19(1), 808, 1-17.

Penders, J., Vink, C., Driessen, C., London, N., Thijs, C., Stobberingh, E.E., 2005. Quantification of Bifidobacterium spp., *Escherichia coli* and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol Lett. 243, 141-147.

Pouteau, E., Kabir-Ahmadi, M., Noah, L., Mazur, A., Dye, L., Hellhammer, J., Pickering, G., Dubray, C., 2018. Superiority of magnesium and vitamin B6 over magnesium alone on severe stress in healthy adults with low magnesemia: A randomized, single-blind clinical trial. Plos One 13(12), 1-17.

Prior, R., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53, 4290-4302.

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

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

Rossi, L., Vagni, S., Polidori, C., Alborali, G.L., Baldi, A., Dell'Orto, V., 2012. Experimental Induction of Escherichia *coli* Diarrhoea in Weaned Piglets. Open Journal of Veterinary Medicine 2, 1-8.

Smirnova, O., Efimova, I., Khil'ko, S., 2012. Antioxidant and Pro-oxidant Activity of Ascorbic and Humic Acids in Radical-Chain Oxidation Processes. Russian Journal of Applied Chemistry 85, 252-255.

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

Trckova, M., Lorencova, A., Babak, V., Neca, J., Ciganek, M., 2017. Effects of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on the health, oxidative stress status and fatty acid profile in weaned piglets. Veterinární medicína 62(1), 16-28.

Trckova, M., Lorencova, A., Hazova, K., Zajacova, Z., 2015. Prophylaxis of post-weaning diarrhoea in piglets by zinc oxide and sodium humate. Veterinarni Medicina 60, 351-360.

Verma, H., Garg, R., 2017. Effect of magnesium supplementation on type 2 diabetes associated cardiovascular risk factors: a systematic review and meta-analysis. Journal of human nutrition and dietetics 30(5), 621-633.

Wang, J., Wu, C., Feng, J., 2011. Effect of dietary antibacterial peptide and zinc-methionine on performance and serum biochemical parameters in piglets. Czech Journal of Animal Science 56, 30-36.

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

Yang, Q., Huang, X., Zhao, S., Sun, W., Yan, Z., Wang, P., Li, S., Huang, W., Zhang, S., Liu, L., Gun, S., 2017. Structure and Function of the Fecal Microbiota in Diarrheic Neonatal Piglets. Front Microbiol. 8(502), 1-13.

Yuan, X., Wang, J., Zhu, X., Zhang, Z., Ai, Y., Sun, G., Wang, Z., Liu, G., 2011. Effect of Copper on Levels of Collagen and Alkaline Phosphatase Activity from Chondrocytes in Newborn Piglets *In Vitro*. Biological Trace Element Research 144, 597-605.

Zhang, J., Lv, Y., Tang, C., Wang, X., 2013. Effects of dietary supplementation with palygorskite on intestinal integrity in weaned piglets. Applied Clay Science 86, 185-189.

Zhao, J., Harper, A.F., Estienne, M.J., Webb Jr, K.E., McElroy, A.P., Denbow, D.M., 2007. Growth performance and intestinal morphology responses in early weaned pigs to supplementation of antibiotic-free diets with an organic copper complex and spray-dried plasma protein in sanitary and nonsanitary environments. Journal of Animal Science 85(5), 1302-1310.

Preeliminary data experiment 4: Mint oils and their in vitro anti-inflammatory effects tested in porcine alveolar macrophages

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7.1. Abstract

Essential oils as feed additives are being investigated for promoting health in piglets due to their anti-inflammatory activity. The objective of the study was to measure the *in vitro* anti-inflammatory effects of peppermint oil and spearmint oil with porcine alveolar macrophages as host immune responses. Briefly, macrophages were harvested from the bronchial lavage of 6 pigs at 6 weeks of age, and then seeded into 24-well plate with at 10^6 cells/mL. After 24 h incubation at 37°C and 5% CO_2, cells were treated with mint oil or lipopolysaccharide (LPS) by randomized complete block design with 12 replicates. The treatments were 2 × 5 factorial arrangements with 2 doses of LPS (0 or 1 μg/mL) and 5 doses of mint oil (0, 25, 50, 100, 200 μg/mL). The supernatants were collected after another 24 h incubation to measure the concentration of tumor necrosis factor alpha (TNF-α) by ELISA assay. Cell viability was also tested by the MTT assay. Data were analyzed using the MIXED procedure of SAS 9.4. Administration of both mint oils and LPS did not impact the PAM cell viability of macrophages. LPS challenge significantly stimulated (P<0.05) TNF-α secretion from macrophages. In the non-challenge group, peppermint oil reduced (P < 0.05) TNF-α production at 25, 50, and 100 μg/mL, whereas spearmint oil reduced (P < 0.05) TNF-α concentration from 50 to 200 μg/mL. In the LPS challenge group, both mint oils linearly inhibited (P<0.001) TNF-α secretion from LPS-challenged macrophages with 200 μg/mL as the strongest dose. Results of the current study indicated that both peppermint and spearmint oils had anti-inflammatory activities *in vitro*. *In vivo* animal trials will be conducted to evaluate their impacts on animal health and performance.

CHAPTER 5

Brief introduction of the scientific works of bioaccumulation of heavy metals from wastewater

Animals should be fed in accordance with the nutritional ecology strategy because livestock nutrition plays a pivotal role in controlling environmental pollution of heavy metals. In swine farms, heavy metal contamination of wastewater considerably reduces its potential recycling for irrigation. However, if the nutritional ecology strategy is not sufficient to reduce the wastewater pollution from livestock production, to ensure water conservation, the development of efficient, cost-effective, reliable materials and methods such as plant-based strategies are needed. Promising possibility to reduce excessive output of metals from livestock wastewater is the process of phytoremediation. This approach is simple clean-up technology which removes, degradates and immobilizes metals from different matrices such as sludge, soil, sediments, groundwater and wastewater through the use of plants that accumulate large amounts of heavy metal contaminants. Thus, in this chapter, I focused to assess the ability of two aquatic species, *Typha latifolia* (Broadleaf cattail) and *Thelypteris palustris* (Marsh fern), to remove zinc and copper from contaminated livestock wastewaters.

Experiment 5:

Bioaccumulation of heavy metals from wastewater through a *Typha latifolia* and *Thelypteris palustris* phytoremediation system

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8.1. Abstract

Animal production is a source of heavy metals in livestock wastewater and also a key link in the food chain, with negative impacts on human and animal health. In intensive animal production systems, the most critical elements are zinc and copper. In order to development of innovative non-invasive strategies to reduce the environmental impact of livestock, this study assessed the ability of two plants, *Typha latifolia* and *Thelypteris palustris*, to bioaccumulate the heavy metals used in animal nutrition, from wastewater. Four mesocosms (width 2.0 m, length 2.0 m, 695 L of water, 210 kg of soil) were assembled outdoors at the Botanical Garden. Two of them were planted with *T. latifolia* (TL treated, n=30; TL control, n=30) and two with *T. palustris* (TP treated, n=60; TP control, n=60). In T0 a solution of a mineral additive premix (Zn 44.02 mg/L; Cu 8.63 mg/L) was dissolved in the treated mesocosms. At T0, d 15 (T1) and d 45 (T2) samples of roots, leaves, stems, soil and water were collected, dried, mineralized and analyzed using ICP-MS in order to obtain HMs content. We found that *T. latifolia* and *T. palustris* accumulate and translocate Zn, Cu from contaminated wastewater into plant tissues in a way that is directly related to the exposure time (T2 for Zn: 271.64±17.70, 409.26±17.70 for Cu: 47.54±3.56, 105.58±3.56 mg/kg of DM, respectively). No visual toxicity signs were observed during the experimental period. This phytoremediation approach could be used as an eco-sustainable approach to counteract the output of heavy metals.

8.2. Introduction

The contamination of wastewater with heavy metals and metalloids (HMs) has become a worldwide concern in areas of intensive agriculture (Bhargava et al. 2012). The long-term consequences of the accumulation of HMs can reduce the quality of cultivation and increase the pollution of agricultural lands (Gul et al. 2015; Jakubus et al. 2013; Liu et al. 2018; Lopez-Alonso et al. 2012; Rossi et al. 2013, 2014 a,b). The major routes of HMs into the soil include atmospheric deposition, agrochemicals, inorganic fertilizers and also animal manure, the latter reflecting the content of HMs from animal feed (Nicholson et al. 2003).

Animal production is thus a possible source of HMs which can contaminate the food chain with a negative impact on human and animal health (Dumont et al. 2012; Jarup 2003; Lyubenova et al. 2013; Ma et al. 2016; Hejna et al. 2019). HMs can enter the animals' diet both as contaminants or undesirable substances and as essential nutrients (Fink-Gremmels 2012; Hejna et al. 2018). Elements such as cobalt (Co), copper (Cu), iron (Fe), iodine (I), manganese (Mn), molybdenum (Mo), selenium (Se) and zinc (Zn) are some of the numerous enzymes that coordinate biological processes, and consequently should be integrated into the animal diet as mineral additives by respecting the maximum admitted level (EC N° 1831/2003; Lopez-Alonso et al. 2012).

The previous study showed that the content of HMs in manure reflected their concentration in the diet (Hejna et al. 2019), and that Zn and Cu, widely used in high doses to control enteric bacterial infections as well as to enhance the integrity of the immune system (Liu et al. 2018) represent the most critical output from swine livestock.

The scenario of livestock has changed significantly in the last decade. In fact, after the antibiotics ban in 2006 in Europe (EC, Reg. 1831/2003), there was an increased use of high dosages of zinc salts, possible after veterinary prescription as an alternative to in-feed antibiotics to control the enteric disease in the growing phases. Despite the antibacterial activity of zinc salts, the use of zinc in feed might have contributed to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). There is worldwide concern that MRSA has become a zoonotic pathogen in animal production. For these reasons together with the environmental issues, the EU has banned the inclusion of pharmacological levels of ZnO after 2022 (EMA/394961/2017).

Since the bioavailability of mineral sources is limited and they are partially absorbed by organisms, the excess is eliminated by excretion. In swine farms, wastewater-derived conventional techniques of civil and livestock waste, could be valuable for agricultural irrigation; however, HM contamination (Chardon et al. 2012; Hejna et al. 2018; Moral et al.

2005; Nicholson et al. 2003) drastically reduces their potential use in irrigation. Since the use of contaminated irrigation water would be responsible for the distribution of large numbers of metallic ions in the environment, the removal of HMs from manure wastewaters is essential in order to improve the soil quality (Gul et al. 2015; Jakubus et al. 2013; Liu et al. 2018; Lopez-Alonso et al. 2012).

Thus, the aim of this study was to assess the ability of two aquatic species, Typha latifolia (Broadleaf cattail) and Thelypteris palustris (Marsh fern), to remove Zn and Cu from contaminated livestock wastewaters, given that these species have already been used to decontaminate water and soils from metals (Chandra and Yadav, 2010; Hazra et al. 2015; Manios et al. 2003a, b; Salem et al. 2017). Cattail is a wetland specie that can be grown under different climatic conditions such as brackish and polluted water and because of their rapid growth and easily harvesting they can be used in phytoremediation (Milam et al. 2004; Ahmad et al. 2017; Rodriguez-Hernandez et al. 2017). Marsh fern also could be ideal aquatic plant for phytoremediation due to its wide range of habitat and easy of cultivation in many environments including agricultural sites, endangered coastal wetlands and urban brownfield sites (Anderson et al. 2007). A phytoremediation pilot mesocosms system was developed, which could be easily managed in animal production systems. In addition, to enable plants to work in the system for a long time and to reduce the amount of exhausted plants that need disposing of a mineral additive premix was dissolved in the wetland water to obtain a concentration of zinc fourteen times higher than the regulation limit.

8.3. Material and methods

8.3.1. Plant culture

A pilot wetland system containing four mesocosms (width: 2.0 m; length 2.0 m; depth 1.2 m) was assembled outdoors at the Botanical Garden of the University of Milan (Italy). The mesocosms were aligned in one row parallel to the sun's pathway to receive the same intensity of light radiation. Each mesocosm had a constant flow-through capacity by a horizontal submerged flow system, which was combined with the open input-output pipe.

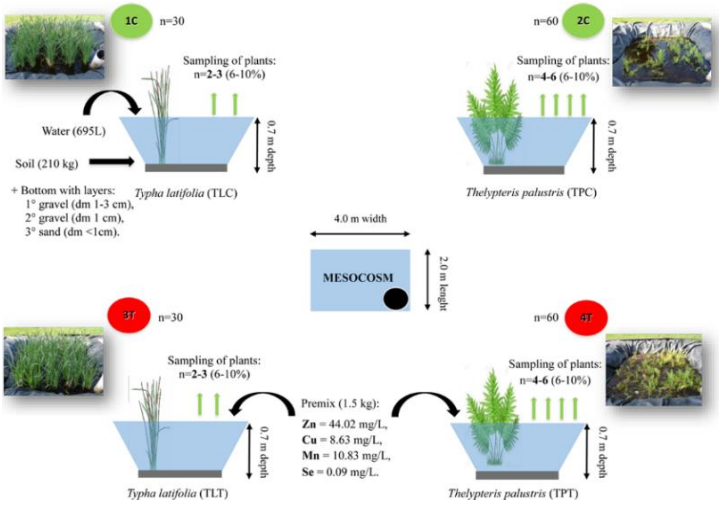
Mesocosms were filled by waterproof cloths, two layers of stone chippings (1st gravel with diameter 1-3 cm; 2nd gravel with diameter 1 cm) and sand (diameter <1cm) was poured into the basis. In order to create positive drainage, gravel was placed, and compacted on the bottom. This substratum was then induced to create a sediment upon water addition, and finally 210 kg of loam for plant culture composed of acid peat, pumice, clay and manure NPK (0.3 s/m

of electric conductibility, 300 kg/m^3 dry density and 90% v/v total porosity; mature commercial compost Flox Containerpflanzez, Blumenerde VitaFlor) was layered on the substratum.

The commercial compost used in the experimental trial contained 45.45% of ashes as fresh weight (f.w.) with 8.57% humidity (Supplementary Table 2). Fresh water (650 L) was added to each mesocosm. Then young healthy plants (purchased from Centro Flora) were planted and were left in the substrate for one week for the adaptation. Two mesocosms were used for *T. latifolia* (TL control: control, n=30; TL treated: treatment, n=30) and two mesocosms were used to test *T. palustris* (TP control: control, n=60; TP treated: treatment, n=60).

After the adaptation (T0), 1.5 kg of a mineral commercial additive premix (feed Maxi CRC 0.5%, Alpha, Zn 20.400 mg/kg, Cu: 4.000 mg/kg, Mn: 5.020 mg/kg, Se 41 mg/kg, Vitamin K: 150 mg/kg; Vitamin B2: 440 (mg/kg); Vitamin A: 1.100.000 Ul/kg; Vitamin D3: 220.000 Ul/kg) was dissolved, more than 14 times higher concentration for Zn referring to the maximum admitted level established by Italian regulation (for Zn: 3 mg/L according D. 337 152/2006 and for Cu: 2 mg/L according 98/83/EC).) in the treatment mesocosms planted with *T. latifolia* (TL treated) and *T. palustris* (TP treated), respectively. The mineral commercial additive premix contains all essential trace elements and macronutrients for animal diet and it is normally added to the feed. The theoretical final concentrations were calculated: 44.02 mg/L of Zn; 8.63 mg/L of Cu (Figure 18). The mineral premix was added carefully to the surface of the water taking care not to spill outside the mesocosm.

Figure 18. The outdoor mesocosms with the amount of mineral additive premix dissolved on the first d of the experiment (T0).



1C: *T. latifolia* control mesocosm; 2C; *T. palustris* control mesocosm; 3T: *T. latifolia* treated mesocosm; 4T: *T. palustris* treated mesocosm.

8.3.2. Plants, soil and water sampling

The experiment took place over a period of 10 weeks. Before the sampling procedure, each mesocosm was separated into three homogenous areas and plants were then collected from these three areas. At T0 and 15 d later (T1), and 45 d later (T2), samples of plants (aerial – leaves/stem and subaerial – rhizomes/roots organs), samples of water (5 mL) and soil (300 g) were collected. A total of 70% of each soil sample were collected near to the plants' roots, and the remaining 30% were collected from the different mesocosm parts. The water samples were derived from the horizontal submerged flow system, and were then combined with a special pipe in order to proceed with the sampling process. The plants were collected from three different mesocosm regions (n=2-3 of *T. latifolia* and n=4-6 of *T. palustris*; around 5-10% of total amount) at T0, T1 and T2. Each plant collected was rinsed twice with the distilled water in order to wash off any soil particles.

8.3.3. Chemical composition of plant samples

The dry matter (DM) of plants (subaerial organs and aerial organs separately, TL control n=14; TL treated n=28; TP control n=14; TP treated n=28) was obtained by inserting the samples in preweighed aluminum bags which were dried in a forced-air oven at 80°C for 72 h (AOAC 2005 method; proc. 930.15; CR No. 152/2009). All dried plants were ground with a laboratory mill to 0.5 mm (Cyclone Sample Mill, Model 3010-019, pbi International, Milan, Italy) and were evaluated from two time experimental points (T0 and T2). Crude protein (CP) was measured following the Kjeldahl method (AOAC 2005 method, proc. 2001.11). Crude fiber (CF) was determined by the Filter Bag technique (AOCS 2005 method, proc. Ba 6a-05). Lipid content (EE) was measured by the Soxhlet method, with prior hydrolysis (European Commission Regulation No. 152/2009). Ashes were measured using a muffle furnace at 550°C (AOAC 2005 method; proc. 942.05). The amylose ratio in starch, on a dry weight basis (DW) was calculated (Megazyme total starch kit) by spectophotometric evaluation at 510 nm.

8.3.4. *Evaluations of HMs in plants, soil and water samples by inductively coupled plasma mass spectrometry (ICP-MS)*

A total of 0.3g of each dried plant (subaerial organs and aerial organs separately, TL control n=14; TL treated n=28; TP control n=14; TP treated n=28) and 0.3 g of dried soil (0.3 g/DM of each; TL control n=6; TL treated n=6; TP control n=6; TP treated n=6) were mineralized by an ultrawave single reaction chamber microwave digestion system (Anton Paar MULTIWAVE 3000) in Teflon tubes filled with 10 ml of HNO_3 (65% concentrated) by applying a one-step temperature ramp (at 120°C in 10’ and maintained for 10). The mineralized samples were cooled for 20 min and the homogenous samples solutions were transferred into

polypropylene test tubes. Plant samples (250 µl) were then diluted 1:40 with a standard solution containing an internal standard (100 µl) and H_2O (9.75 ml). The soil samples (100 µl) were diluted 1:100 with a standard solution containing an internal standard (100 µl) and HNO_3 (0.3 M, 10 ml). Water samples were analyzed without dilution (5,0 mL; TL control n=4; TL treated n=4; TP control n=4; TP treated n=4).

An aliquot of 2 mgL^{-1} of an internal standard solution (^{72}Ge, ^{89}Y, ^{159}Tb) was added to the samples and calibration curve to obtain a final concentration of 20 μgL^{-1}. All samples were analysed in triplicate by inductively coupled plasma mass spectrometry (ICP-MS; Bruker Aurora M90 ICP-MS, Bremen, Germany) in order to detect the following elements: Na, Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, and Pb (Supplementary Tables 3 and 4). The accuracy of the results obtained using ICP-MS was evaluated using internal reference materials supplied by LGC Standards Company: sewage sludge (LGC 61812); poultry feed (LGC7173); and waste water (SPS-WW2 1). The typical polyatomical analysis interferences were removed using the collision-reaction interface (CRI) with an H_2 flow of 75mL/min^{-1} through a skimmer cone.

8.3.5. Statistical analysis

In order to evaluate any statistically significant differences among mean values, all data were analyzed using Glimmix of SAS software (9.4., SAS Inst. Inc., Cary, NC). The analysis accounted for the fixed effects of treatment, time, plant type and part, and associated two-way interactions and the random effect of plant (treatment). Repeated measures were used as the time (treatment). Means were considered different when $P < 0.05$ and tended to differ if $0.05 < P \leq 0.10$. Tukey-Kramer studentized adjustments were used to separate treatment means within the two-way interactions. Within significant two-way interactions, the slice option was used to separate means within a specific time and plant type. The results are reported as least squares means and standard errors of the means.

8.4. Results

8.4.1. Biomass and chemical composition of plants

In order to detect the effect of Zn and Cu exposure on plant growth, the amount of dry matter (DM)/plant was measured as an indicator of the biomass. *T. latifolia* and *T. palustris* plants grew normally in the control and metal-treated mesocoms from T0 to T2 as showed increasing trend of the plant biomass (Table 25). In fact, with respect to T0, the growth rate for TL treated and TP treated was higher (504.17% and 183.33%, respectively) comparing to TL

control and TP control (329.63% and 131.58%, respectively). Interestingly, for both species, the biomass mostly increased in T2 treated with respect to control mesocosms.

Table 25. The chemical composition (on DM basis) and the biomass of *T. latifolia* and *T. palustris* plants (for aerial organs and subaerial organs) in time points (T0 and T2) for control (TL control) and treatment (TL treated) mesocosms.

Chemical composition								
Part of plants	Treatment	Time points	Humidity (%)	Crude protein (g/100g)	Crude fiber (g/100g)	Lipids (g/100g)	Ash (g/100g)	Starch (g/100g)
T. latifolia								
Aerial organs	Control	T0	21.04	5.34	17.42	2.19	9.41	19.43
		T2	10.05	8.56	32.71	2.52	9.75	10.81
	Treated	T0	13.86	10.78	17.92	2.93	9.36	19.90
		T2	8.83	11.54	29.04	2.60	10.70	10.12
Subaerial organs	Control	T0	12.53	2.97	26.43	0.94	7.27	-
		T2	8.33	4.96	26.01	0.91	9.82	-
	Treated	T0	9.53	4.49	26.31	1.05	7.73	-
		T2	6.55	5.62	26.78	0.78	10.54	-
Biomass of *T. latifolia* (kg DM/Plant)								
Control		T0	0.027±0.010					
		T2	0.089±0.054					
Treated		T0	0.024±0.010					
		T2	0.121±0.075					
T. palustris								
Aerial organs	Control	T0	24.85	7.69	24.51	1.68	6.75	8.90
		T2	16.03	7.92	27.08	1.84	8.87	8.09
	Treated	T0	23.88	7.26	24.25	1.76	7.23	6.02
		T2	22.20	10.20	29.26	1.27	9.01	12.53
Subaerial organs	Control	T0	18.72	5.48	20.93	1.23	6.68	18.07
		T2	14.92	6.81	23.26	0.55	9.06	20.67
	Treated	T0	15.31	4.46	21.54	3.66	8.71	22.32
		T2	13.34	11.46	27.01	0.72	10.61	16.30
Biomass of *T. palustris* (kg DM/Plant)								
Control		T0	0.019±0.011					
		T2	0.025±0.018					
Treated		T0	0.018±0.011					
		T2	0.033±0.014					

T0: first d of the experiment, T2: 45 d later.

The principal chemical components of the control and treated plants (aerial organs and subaerial organs) are presented in Table 25 for *T. latifolia* and *T. palustris*. For both plants, fiber and ash content increased from T0 to T2 in all the organs in parallel to the decrease in water content. However, slight differences were observed in plants grown in the control with respect to the treated mesocoms. On the other hand, there was a decrease in total lipids in *T. latifolia* and *T. palustris* after 45 d of metal exposure compared to the control (see T2 with

respect to T0; Table 25). In the control leaves of both plants, lipids slightly increased from T0 to T2, while in treated samples there was a decrease of about 10% and 25% for *T. latifolia* and *T. palustris*, respectively. In rhizome, a decrease of total lipids was observed both in the control and treated plants particularly in marsh ferns (Table 25; compare T0 and T2). However, in T2 there was a greater decrease of lipids in the treated plants than in the control plants (Table 25).

Proteins showed different trends in *T. latifolia* and *T. palustris*, although they increased in both plants from T0 to T2. This increase was more pronounced in marsh ferns than in Monocot plants. The quantification of starch showed that the amount of this polymer was different depending on the organ or the plant. In *T. latifolia*, no differences were observed for aerial organs in the treated and control samples (Table 25; see T2 with respect to T0). An opposite trend of starch content was observed in the aerial and subaerial organs of *T. palustris* after metal exposure with respect to the control. In fact, in the control, starch decreased in leaves and increased in rhizomes during plant growth (compare T0 and T2). On the other hand, in T2 treated plants, an increase of starch in leaves was accompanied by a decrease of starch in the subaerial organs with respect to T0 (Table 25).

8.4.2. Content of Cu^{2+} and Zn^{2+} in plants, soil and water from T. latifolia plants by ICP-MS

To evaluate the ability of *T. latifolia* to accumulate Zn and Cu and thus phytoremediate contaminated water, the concentrations of metals in plants, water and soil were measured in T0, T1 and T2.

In control mesocoms, whole *T. latifolia* plants showed the same concentration of Zn^{2+} and Cu^{2+} in samples collected in T0, T1 and T2 (Table 26). The same behavior was observed when subaerial and aerial organs were considered separately. However, there was an increase of Cu^{2+} in subaerial organs of T2 controls plants, although not significant ($p < 0.05$), showing that root and rhizomes can accumulate Cu^{2+}, which was naturally present in the soil and water. In treated samples, the plants began to accumulate Zn^{2+} and Cu^{2+} after 15 d of metal exposure, since there was only a significant increase in metal concentration in T2 (Zn: $p < 0.001$; TL treated = 271.64±17.71 vs. TL control = 55.79±17.71 mg/kg; Cu: $p < 0.001$; TL treated =47.54±3.56 vs. TL control =15.20±3.56 mg/kg; Table 26).

However, even if no significantly different was observed for the Zn and Cu concertation in aerial and subaerial organs, Zn was accumulated in TL treated subaerial organs, with the maximum concentration at T2 (177.28±30.66 mg/kg). At the same time, TL control showed a concentration of zinc of about 77.16±30.66 mg/kg. Similarly, the Zn concentration of aerial

organs was higher in T2-TL treated than T2-TL control (59.29±30.66 vs. 31.26±30.66 mg/kg, respectively).

Table 26. The average Zn and Cu concentration in *T. latifolia* (TL) plants in the control and treatment mesocosms (TL control; TL treated) at the three time points (T0, T1, T2).

Experimental groups	Time point	Concentrations of heavy metals (mg/kg DM)	
		Zn	Cu
TL control	T0	56.35±17.70^{aA}	12.64±3.56^{aA}
	T1	57.61±17.70^{aA}	10.47±3.56^{aA}
	T2	55.79±17.70^{aA}	15.20±3.56^{aA}
TL treated	T0	81.14±17.70^{aA}	13.81±3.56^{aA}
	T1	105.80±17.70^{aA}	25.92±3.56^{aA}
	T2	271.64±17.70^{bB}	47.54±3.56^{bB}

TL control: *T. latifolia* control mesocosm; TL treated: *T. latifolia* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.
a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control and TL treated ($p < 0.001$).

Higher Cu concentrations were also observed in aerial and subaerial organs of metal treated plants with respect to the control. In addition, rhizomes/roots showed a higher Cu content compared with aerial organs (33.29±6.16 vs. 14.73±6.16 mg/kg, respectively.

The increase of Zn^{2+} and Cu^{2+} concentrations in plant organs was related by a decrease of these metals in water (Table 27). Zn^{2+} and Cu^{2+} were higher in the water of T0 treated mesocoms with respect to the controls due to the addition of the commercial mineral additive premix containing metals used in the experimental trial. The metals in the water had already decreased after two weeks (T1, Table 27) remaining constant for Zn, and slightly decreasing for Cu in T2 water. The decrease of metals in water was in parallel with the increase of metals in soil, particularly in T2 samples (Table 27; $p < 0{,}001$).

Since the bioavailability of metals depends on the pH in the environment, the pH values has been measured. During the experiment, the pH of water varied from neutral to slightly alkaline. However, even if no significantly different, in TL control the pH values remained higher with respect to TL treated mesocosm (Table 27). Moreover, the mineral additive premix inclusion led to a reduction of pH at the beginning of the experiment (T0 7.36 vs 7.00).

Table 27. The average Zn and Cu concentration in soil and water and pH of water of *T. latifolia* (TL) mesocosms in the control and treatment mesocosms (TL control; TL treated) in the three time points (T0, T1, T2).

Experimental groups	Time points	Concentration of heavy metals (mg/kg)	
		Zn	**Cu**
TL control soil	T0	59.19 ± 30.66^{aA}	8.88 ± 6.16^{aA}
	T1	46.01 ± 30.66^{aA}	6.10 ± 6.16^{aA}
	T2	58.94 ± 30.66^{aA}	6.71 ± 6.16^{aA}
TL treated soil	T0	87.18 ± 30.66^{aA}	12.14 ± 6.16^{aA}
	T1	179.72 ± 30.66^{aA}	29.97 ± 6.16^{aA}
	T2	578.36 ± 30.66^{bB}	94.59 ± 6.16^{bB}
Concentration of heavy metals (mg/L)			
TL control H_2O	T0	0.001	0.009
	T1	0.001	0.004
	T2	0.005	0.007
TL treated H_2O	T0	0.187	0.204
	T1	0.023	0.033
	T2	0.022	0.024
pH of H_2O			
TL control	T0	7.36 ± 0.07^{a}	
	T1	7.14 ± 0.07^{a}	
	T2	7.58 ± 0.07^{a}	
TL treated	T0	7.00 ± 0.07^{a}	
	T1	7.07 ± 0.07^{a}	
	T2	7.25 ± 0.07^{a}	

TL control: *T. latifolia* control mesocosm; TL treated: *T. latifolia* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control and TL treated ($p < 0.001$); for pH: $p < 0.05$.

8.4.3. Content of Cu^{2+} and Zn^{2+} in plants, soil and water from T. palustris plant by ICP-MS

As observed in *T. latifolia*, whole plants of *T. palustris* were also able to accumulate Zn^{2+} and Cu^{2+} in their organs. In fact, higher concentrations of metals were detected in TP treated than in the control already 15 d (T1) after metal addition (Table 28; $p < 0.001$) and there was a slight decrease in T2 plants. There was a similar trend in the aerial and subaerial organs separately, in which high concentrations of Zn^{2+} and Cu^{2+} were reached in T1-TP treated samples (Table 29). Zn was mostly accumulated in TP treated subaerial organs, with the maximum concentration at T2 (Table 29). At T2, the Zn concentration of aerial organs was higher in TP treated than TP control (Table 29). Cu concentration also significantly increased

in T1 and T2-TP treated subaerial organs compared with TP control (Table 29; $p < 0,001$), and likewise for T1-TP treated aerial organs. Surprisingly, 45 d after metal addition, Cu decreased significantly in leaves of *T. palustris* (Table 29, T2). Translocation of metals from subaerial organs to leaves was higher with respect to *T. latifolia*, however, *T. palustris* accumulated Zn^{2+} and Cu^{2+} preferentially in subaerial organs (Table 29).

Table 28. The average Zn and Cu concentration in *T. palustris* (TP) plants in the control and the treatment mesocosms (TP control; TP treated) in the three time points (T0, T1, T2).

Experimental groups	Time point	Concentrations of heavy metals (mg/kg DM)	
		Zn	Cu
TP control	T0	113.33 ± 17.70^{aA}	11.30 ± 3.56^{aA}
	T1	85.62 ± 17.70^{aA}	18.25 ± 3.56^{aA}
	T2	88.36 ± 17.70^{aA}	16.50 ± 3.56^{aA}
TP treated	T0	89.11 ± 17.70^{aA}	12.46 ± 3.56^{aA}
	T1	414.67 ± 17.70^{bB}	136.12 ± 3.56^{bB}
	T2	409.26 ± 17.70^{bB}	105.58 ± 3.56^{bB}

TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control and TL treated ($p < 0.001$).

Table 29. The average Zn and Cu concentration in *T. palustris* (TP) subaerial organs and the average Zn and Cu concentration in *T. palustris* aerial organs in the control and in the treatment mesocosms (TP control; TP treated) in the three time points (T0, T1, T2).

Experimental groups	Time point	Concentrations of heavy metals (mg/kg DM)	
		Zn	Cu
T. palustris aerial organs			
TP control	T0	35.49 ± 30.66^{aA}	7.08 ± 6.16^{aA}
	T1	43.95 ± 30.66^{aA}	8.94 ± 6.16^{aA}
	T2	22.04 ± 30.66^{aA}	8.98 ± 6.16^{aA}
TP treated	T0	$22.32 \pm 30.66b^{aA}$	6.59 ± 6.16^{aA}
	T1	235.08 ± 30.66^{bB}	119.48 ± 6.16^{bB}
	T2	201.63 ± 30.66^{bB}	33.03 ± 6.16^{aA}
T. palustris subaerial organs			
TP control	T0	191.96 ± 30.66^{aA}	$15,21 \pm 6.16^{aA}$
	T1	93.94 ± 30.66^{aA}	31.79 ± 6.16^{aA}
	T2	134.51 ± 30.66^{aA}	24.19 ± 6.16^{aA}
TP treated	T0	175.79 ± 30.66^{aA}	18.12 ± 6.16^{aA}
	T1	527.37 ± 30.66^{bAB}	204.70 ± 6.16^{bB}
	T2	786.49 ± 30.66^{bB}	235.10 ± 6.16^{bB}

TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control and TL treated ($p < 0.001$).

The increase of metals in plants was related by a decrease of Zn^{2+} and Cu^{2+} in water (Table 30). As observed in *T. latifolia* mesocoms, Zn^{2+} and Cu^{2+} were higher in T0 treated water than in the controls; during the experimental proceed the metal concentration decreased both in T1 and T2 samples (Table 30). Unlike the *T. latifolia* mesocoms, both Zn^{2+} and Cu^{2+} were present at significantly higher concentrations in soil two weeks after the metals had been added (Table 30; $p < 0.001$). There was then a significant decrease in the Zn^{2+} and Cu^{2+} concentration in T2 soil samples (Table 30; $p < 0.001$), confirming the idea that the uptake of metals by plants occurs preferentially by soil.

Table 30. The average Zn and Cu concentration in soil and water and pH of water of *T. palustris* (TP) mesocosms in the control and treatment mesocosms (TL control; TL treated) in the three time points (T0, T1, T2).

Experimental groups	Time points	Concentration of heavy metals (mg/kg)	
		Zn	**Cu**
TP control soil	T0	112.53 ± 30.66^{aA}	11.60 ± 6.16^{aA}
	T1	118.97 ± 30.66^{aA}	14.02 ± 6.16^{aA}
	T2	108.55 ± 30.66^{aA}	16.34 ± 6.16^{aA}
TP treated soil	T0	69.24 ± 30.66^{aA}	12.66 ± 6.16^{aA}
	T1	481.55 ± 30.66^{bB}	84.17 ± 6.16^{bB}
	T2	239.65 ± 30.66^{aA}	48.60 ± 6.16^{aA}
Concentration of heavy metals (mg/L)			
TP control H_2O	T0	0.001	0.007
	T1	0.001	0.003
	T2	0.002	0.005
TP treated H_2O	T0	0.381	0.240
	T1	0.053	0.025
	T2	0.036	0.013
pH of H_2O			
TP control	T0	7.18 ± 0.03^{a}	
	T1	7.12 ± 0.03^{a}	
	T2	7.29 ± 0.03^{a}	
TP treated	T0	6.99 ± 0.03^{a}	
	T1	7.27 ± 0.03^{b}	
	T2	7.41 ± 0.03^{a}	

TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control and TL treated ($p < 0.001$); for pH: $p < 0.05$.

During the experiment, water pH varied from neutral to slightly alkaline both in the control and in treated mesocoms. After the premix had been added in T0 the pH decreased in TP treated compared with TP control. However, even if later pH mostly increased in T1-TP~~T~~ treated and T2-TP treated with respect to T1-TL treated T2-TL treated mesocoms (Table 30, $p < 0.05$ in T1).

8.5. Discussion

The intensive animal production system is a source of HM input into environment and also a key link in the food chain. This has led to the development of approaches to increase the sustainability of intensive livestock farming. Animal manure reflects the composition of their diet and is frequently used as an organic fertilizer given that it contains a broad range of nutrients such as nitrogen, phosphorus, potassium, as well as micronutrients and HMs. Although the maximum permitted levels are well defined by EU regulations (EC N° 1831/2003), they are often above the physiological requirements.

In line with the major topics of agroecology, multidisciplinary strategies are required that take into account the needs of animals (health, welfare and nutrition productivity) and farmers (profitability and productivity) together with the environment. Phytoremediation system is used to refine pre-treated wastewaters before they are used for irrigation (Peterson, 1998).

The tolerance threshold for HM accumulation in the tissues in each plant differs from species to species and is determined by genetical, environmental and physiological features (Ali et al. 2013; Lone et al. 2008; Mukhopadhyay et al. 2010; Thangavel et al. 2004). However, our approach showed that both *T. palustris* and *T. latifolia* removed Zn and Cu from pilot wetland systems contaminated by a mineral additive premix normally used in animal diets.

8.5.1. T. latifolia and T. palustris could work in series to refine wastewater by Cu and Zn phytoremediation

The ability of *T. latifolia* to accumulate metals is well known (Fediuc and Erdei 2002; Hemmati et al. 2012; Klink et al. 2013; Klink et al. 2016; Klink 2017; Kumari and Tripathi, 2015; Lyubenova and Shroder, 2011; Manios et al. 2002, 2003 a,b; Maric et al. 2013; Peralta et al. 2001; Rafati et al. 2011; Rai et al. 1995; Ye et al. 1997). On the other hand, the potential of T. palustris in phytoremediation systems has only been tested for arsenic (Anderson et al. 2011).

In order to mimic the condition of wastewater refining systems in the livestock, an outdoor pilot wetland system was used. In this system, *T. latifolia* and *T. palustris* showed different capability to accumulated Cu and Zn contained after the mineral additive premix has

been added to the water in the TL treated and TP treated mesocosms. The decreasing trend for Zn and Cu in the water and soil was accompanied by an increase of metal concentration in the TL treated and TP treated plants. Our phytoremediation pilot system decontaminated the wastewater from the toxic elements in line with Petroselli et al. 2015.

Analyses of HM concentrations in plants (in the whole plants or in the aerial and subaerial organs) suggested that T. palustris was more effective than T. latifolia in accumulating metals in subaerial organs and in translocating them to leaves in a short time. The low capacity of T. latifolia to translocate metals is already reported and is considered a metal tolerance strategy (Feriuc end Erdei, 2002; Klink et al. 2013, 2017).

Already after 15 d of exposure to metals, T. palustris was able to efficiently uptake Zn and Cu, while T. latifolia started the accumulation process later. This difference could be due to the high metal concentration in the soil. When we added metals to the water in the treatment mesocoms, Zn and Cu concentrations were higher in the water than in the soil. The concentrations of the metals then decreased in water and increased in soil. It has been reported that in wetlands, the binding of metals to substrate is the major process for water to remove metals (Almeida et al. 2017; Yadav et al. 2012). Our data suggest that metal uptake occurs preferentially by the soil rather than by the water. In addition, the concentrations of Zn and Cu increased earlier in the soil of *T. palustri*s compared to *T latifolia* mesocoms.

It is possible to hypothesize that marsh ferns modify the chemical features of soil by increasing the adsorption capacity of the matrix. In fact, several molecules were released by the roots into the rhizosphere and could thus modify the availability of nutrients and the matrix composition (Dakora and Phillips, 2002; Lyubenova et al. 2013). The significant decrease of metal concentrations in the soil in T2 samples suggested that T. palustris was more efficient in short-term phytoremediation processes. The co-presence of two species which work in series could increase the efficiency of the phytoremediation wetland systems.

In wetland systems, the degree of metal translocation by soil to plants depends on several environmental conditions (Yang and Ye, 2009). The pH influences the bioavailability of metal ions, and low pH promotes metal accumulation in rooted wetland plants (Emamverdian et al. 2015; Yang and Ye, 2009). The optimal condition for the uptake of several nutrients in T. latifolia is a pH value of 6.5 (Brix et al. 2002; Dyhr-Jensen and Brix 1996). The addition of mineral additive led to a decrease in water pH, which during the experiment subsequently increased to slightly alkaline values. This trend has been observed in other phytoremediation systems (Barakat 2011; Han et al. 2015; Kumari et al. 2015) and could be due to the ability of

plants to modify the pH condition in the rhizosphere (Brix et al. 2002; Dyhr-Jensen and Brix 1996). The increase in water pH to slightly alkaline values did not seem to affect plant uptake.

8.5.2. *T. latifolia and T. palustris differently respond to metal exposure in a pilot wetland system*

Our results showed that the metal concentrations used in the pilot system were not toxic for the two plants, in fact the biomass increased over time. Biomass is a relevant factor for metal exchange and an important aspect of the health status of plants. In fact, according to Maric et al. (2013) the ideal plant for removing HMs should have a very large biomass and a rapid growth. Although T. latifolia showed a lower capacity to absorb metals in a short period of time, it may be better than hyperaccumulator plants because it produces more biomass and has a higher growth rate (Ali et al. 2013). Interestingly, in our treated plants the biomass increase was higher with respect to the control suggesting that although the metal concentrations used were fourteen times higher than that permitted by Italian regulations, they stimulate plant growth.

Zn^{2+} and Cu^{2+} are essential trace metals involved in many physiological processes in plants (Arif et al. 2016; Emamverdian et al. 2015; Manios et al. 2002). It is possible to hypothesize that these concentrations provide an amount of heavy metals which accelerates the growth of *T. palustris* and *T. latifolia*. Alternatively, the increase in biomass could be a tolerance mechanism of plants which grow in order to increase the number of tissues where metals could be accumulated or diluted.

Despite the increase of biomass, some chemical variations were recorded by ICP-analyses. Most relevant alterations in treated with respect to T2 control plants were detected for proteins, lipids and starch. The different behaviors of protein content observed in T2-TL treated and T2-TP treated with respect to the control suggested that the early uptake of metals by *T. palustris*, could activate stress and tolerance mechanisms that enabled plants to grow in the contaminated mesocoms. It is known that HMs trigger the expression of those genes that codify for proteins involved in stress responses (Hasan et al. 2017), such as phytochelatins and metallothioneins or enzymes with antioxidant activities to scavenge active oxygen species (REF). These tolerance mechanisms could also be activated in T. palustris during metal exposure.

After metal treatment, in T2 samples, the amount of lipid decreased with respect to the control in both plants. This difference was similar in subaerial organs and in leaves, but appeared more pronounced in marsh ferns compared to T. latifolia. This effect could be due to a lower T. palustris metal tolerance or to a rapid accumulation of metals in this plant (accompanying paper Stroppa et al. 2019). The ability of metals to induce a decrease in lipids

and changes in lipid composition has been reported in other plants (Elloumi et al. 2014; Oves et al. 2016). The reduction of lipids that we detected in T2 metal exposed plants, particularly in T. palustris, could also be due to an alteration in the carbohydrate metabolisms. In fact, in T. palustris, the increase of starch in aerial organs suggests an evolution of chloroplasts into amyloplasts, as also observed in microscopical analyses (accompanying paper Stroppa et al. 2019). Plastids transformation could trigger a reduction in thylakoid and thus a reduction of lipid content. The decrease of starch in roots and rhizomes of both plants was different from what has been reported elsewhere for other plants in phytoremediation systems since in this case the starch content in roots and rhizomes increased (Frossard et al. 1989; Higuchi et al. 2015; Todeschini et al. 2011). The modification of carbohydrate metabolisms was considered a response of plants to metal accumulation. In L. perenne, the increase in Zn induced a fructan accumulation, while the increase in Cu induced an increase of starch (Frossard et al. 1989). In our study, the presence of high amounts of starch in the leaves of *T. palustris*, suggests that it has greater sensitivity to metal exposure than *T. latifolia*. (12.53 vs 10.12 g/100g in T2 treated mesocosms, respectively).

Since these modifications occurred in the absence of visible symptoms of phytotoxicity, it appears that in *T. latifolia* and *T. palustris*, some mechanisms of metal tolerance have been present. However, it is not possible to exclude that some effects to metal exposure could also be due to the toxicity of metals. Further analyses could better clarify this point.

Moreover, tested plants after the bioaccumulation process can be used as eco-material for building constructions (Melià et. al, 2014). Contemporary building materials (cement concrete, steel) require high energy for their production and are responsible for the emission of greenhouse gases (Morel et al. 2001; Venkatarama Reddy and Prasanna Kumar, 2010). The use of natural materials is encouraged by its availability, large quantities, affordable cost and less energy needed during the production process (Melià et al. 2014). Thus, once at the end of life the natural material is recyclable with no impact on the environment (Delgado and Guerrero, 2006).

8.6. Conclusions

The mesocosms treated with *T. latifolia* and *T. palustris* in our experiment were highly contaminated with a heavy metal mineral additive premix widely used in swine nutrition. *T. latifolia* and *T. palustris* exhibited relatively high Zn and Cu accumulation and translocation abilities. In addition, *T. latifolia* and *T. palustris* tolerated high levels of Zn and Cu, with no visual toxicity signs and no significant visual effect on their development throughout the

experimental period. To conclude, both *T. latifolia* and *T. palustris* can accumulate and translocate the Zn and Cu form contaminated wastewater. However, in order to decrease critical amounts of Zn and Cu in swine livestock output, when its level is critical, *T. palustris* can be used to reduce the Zn and Cu content in a short period of time. On the other hand, the wastewater phytoremediation for a long time could be achieved by *T. latifolia* working in series with respect *T. palustris*. The results suggest that the ability of the two plants to survive different concentrations of Zn and Cu indicates that they could be used in a phytoremediation strategy to counteract the output of zinc and copper, and possibly other HMs from the livestock industry.

8.7. References

AOCS, "Official Methods of the American Oil Chemists Society," 5th Edition, American Oil Chemists Society, Champaign, 1998.

DM, "Approvazione dei Metodi di Analisi per il Controllo Ufficiale degli Alimenti per Animali e Soppressione di Altri Metodi Inerenti al Controllo del Medesimo Settore Merceologico," Gazzetta Ufficiale, No. 31, Suppl. 13, 21 Dicembre 1998.

Agenzia per la Protezione dell' Ambiente e per i Servizi Tecnici. Confronto tra concentracioni limite accettabili ex D.M.471/99 e concentrazioni soglia do contaminazione. 2006. ex D/Lgs 152/06.

Ahmad MS, Mehmood MA, Taqvi STH, Elkamel A, Liu CG, Xu J, Rahimuddin SA, Gull M. Pyrolysis, kinetics analysis, thermodynamics parameters and reaction mechanism of *Typha latifolia* to evaluate its bioenergy potential. Bioresour Technol. 2017; 491-501. https://doi.org/110.1016/j.biortech.2017.08.162.

Ali H, Khan E, Anwar Sajad M. Phytoremediation of heavy metals - concepts and applications. Chemosphere. 2013; 869-881; https://doi.org/10.1016/ j.chemosphere. 2013.01.075.

Almeida CMR, Santos F, Ferreira AC, Gomes CR, Basto, MC, Mucha AP. Constructed wetlands for the removal of metals from livestock wastewater - can the presence of veterinary antibiotics affect removals? Ecotoxicol Environ Saf. 2016. 143-148; https://doi.org/10.1016/j.ecoenv.2016.11.021.

Anderson LL, Walsh M, Roy A, Bianchetti CM, Merchan G. The potential of *Thelypteris palustris* and *Asparagus sprengeri* in phytoremediation of arsenic contamination. Int J Phytoremediation. 2011; 177-84; https://doi.org/10.1080/15226511003671346.

Anderson LS, Walsh MM. Arsenic uptake by common marsh fern *Thelypteris palustris* and its potential for phytoremediation. Sci Tot Environ. 2007; 263-265.

Arif N, Yadav V, Singh S, Singh S, Ahmad P, Mishra RK, *et al.* Influence of high and low levels of plant-beneficial heavy metal ions on plant growth and development. Front Environ Sci. 2016; https://doi.org/10.3389/fenvs.2016.00069.

Barakat MA. New trends in removing heavy metals from industrial wastewater. Arab J Chem. 2011; 361-377; https://doi.org/10.1016/j.arabjc.2010.07.019.

Bhargava A, Carmona FF, Bhargava M, Srivastava S. Approaches for enhanced phytoextraction of heavy metals. J Environ Manage. 2012; 103-120; https://doi.org/10.1016/j.jenvman.2012.04.002.

Brix H, Dyhr-Jensen K, Lorenzen B. Root-zone acidity and nitrogen source affects *Typha latifolia* L. growth and uptake kinetics of ammonium and nitrate. J Exp Bot. 2002; 2441-2450; https://doi.org/10.1093/jxb/erf106.

Chandra R, Yadav S. Potential of *Typha angustifolia* for phytoremediation of heavy metals from aqueous solution of phenol and melanoidin. Ecol Eng. 2010; 1277-1284; https://doi.org/10.1016/j.ecoleng.2010.06.003.

Chardon X, Rigolot C, Baratte C, Espagnol S, Raison C, Martin-Clouaire R, *et al*. MELODIE: a whole-farm model to study the dynamics of nutrients in dairy and pig farms with crops. Animal. 2012; 1711-1721; https://doi.org/10.1017/S1751731112000687.

Commission Implementing Regulation (EU) 2016/1095 of 6 July 2016 concerning the authorization of Zinc acetate dihydrate, Zinc chloride anhydrous, Zinc oxide (…) as feed additive for all animal species. Off J. L: 182/7.

Dakora FD, Phillips DA. Root exudates as mediators of mineral acquisition in low-nutrient environment. Plant Soil. 2002; 35-47; https://doi.org/10.1023/A:1020809400075.

Delgado MCJ, Guerrero IC. Earth building in Spain. Constr Build Mater. 2006; 679-690; https://doi.org/10.1016/j.conbuildmat.2005.02.006.

Dumont B, Fortun-Lamothe L, Jouven M, Thomas M, Tichit M. Prospects from agroecology and industrial ecology for animal production in the 21st century. Animal. 2012;1028-1043; https://doi.org/10.1017/S1751731112002418.

Dyhr-Jensen K, Brix H. Effects of pH on ammonium uptake by *Typha latifolia L*. Plant Cell Environ. 1996; 1431-1436; https://doi.org/10.1111/j.1365-3040.1996.tb00022.x.

Elloumi N, Zouari M, L Chaari L, Jomni C, Marzouk B, Abdallah FB. Effects of cadmium on lipids of almond seedlings (*Prunus dulcis*). Bot Stud. 2014, 55:61; https://doi.org/10.1186/s40529-014-0061-7.

Emamverdian A, Ding Y, Mokhberdoran F, Xie Y. Heavy metal stress and some mechanisms of plant defense response. Sci World J. 2015, https://doi.org/10.1155/2015/756120.

European Medicine Agency (EMA) N° 394961/2017. Questions and answers on veterinary medicinal products containing zinc oxide to be administered orally to food-producing species.

European Parliament and the Council. Regulation (EC) no 1831/2003 of 22 September 2003 on additives for use in animal nutrition. Off J. L: 268/29.

Fediuc E, Erdei L. Physiological and biochemical aspects of cadmium toxicity and protective mechanisms induced in *Phragmites australis* and *Typha latifolia*. J. Plant Physiol. 2002; 265-271; https://doi.org/10.1078/0176-1617-00639.

Fink-Gremmels J. Animal Feed Contamination, 1st Edition. Effects on Livestock and Food Safety. Woodhead Publishing Limited, Cambridge 2012.

Frossard R, Stadelmann FX, Niederhauser J. Effects of different heavy metals on fructan, sugar and starch content of ryegrass. J Plant Physiol. 1989; 180-185; https://doi.org/10.1016/S0176-1617(89)80052-5.

Gul S, Naz A, Fareed I, Khan A, Irshad M. Speciation of heavy metals during co-composting of livestock manure. Pol J Chem Technol. 2015; 19-23; https://doi.org/10.1515/pjct-2015-0044.

Han J, Chen F, Zhou Y, Wang C. High Pb concentration stress on *Typha latifolia* growth and Pb removal in microcosm wetlands. Water Sci Technol. 2015; 71 (11): 1734-1741; https://doi.org/10.2166/wst.2015.163.

Hasan K, Cheng Y, Kanwar MK, Chu XY, Ahammed GJ, Qi ZY. Responses of plant proteins to heavy metal stress - a review. Front. Plant Sci. 2017; https://doi.org/10.3389/fpls.2017.01492.

Hazra M, Avishek K, Pathak G. Phytoremedial potential of *Typha latifolia*, *Eichornia crassipes* and *Monochoria hastata* found in contaminated water bodies across Ranchi City (India). Int J Phytoremediation. 2015; 835-840; https://doi.org/10.1080/15226514.2014.964847.

Hejna M, Baldi A, Onelli E, Gottardo D, Pilu SR, Dell'Orto V, *et al*. Evaluation of heavy metals in intensive animal production systems. It J Anim Sc. 2017a; 97.

Hejna M, Gottardo D, Baldi A, Dell'Orto V, Cheli F, Zaninelli M, *et al*. Review: Nutritional ecology of heavy metals. Animal. 2018; 1-15; https://doi.org/10.1017/S175173111700355X.

Hejna M, Moscatelli A, Onelli E, Baldi A, Pilu S, Rossi. Evaluation of concentration of heavy metals in animal rearing system. It J Anim Sc. 2019; https://doi.org/10.1080 /1828051X.2019.1642806.

Hejna M, Stroppa N, Moscatelli A, De Nisi D, Dell'Orto V, Pilu SR, *et al*. Phytoremediation as an innovative approach to control heavy metals output from livestock. It J Anim Sci. 2017b; 128.

Hemmati F, Yazdi Nezhad M. Survey of *Typha Latifolia* for phytoremediation of cadmium in international shadegan wetland. Adv Environ Biol. 2012; 6(12): 4041-4044.

Higuchi K, Kanai M, Tsuchiya M, Ishii H, Shibuya N, Fujita N, *et al*. Common reed accumulates starch in its stem by metabolic adaptation under Cd stress conditions. Front Plant Sci. 2015; 138; https://doi.org/10.3389/fpls.2015.00138.

Jakubus M, Dach J, Starmans D. Biovailability of copper and zinc in pig and cattle slurries. Fresenius Envir Bull. 2013; Vol.22 no 4: 995-1002; https://doi.org/10.2527/jas.53895.

Jarup L. Hazards of heavy metal contamination. Br Med Bull. 2003; 167-82; https://doi.org/10.1093/bmb/ldg032.

Klink A, Macioł A, Wisłocka M, Krawczyk J. Metal accumulation and distribution in the organs of *Typha latifolia L.* (cattail) and their potential use in bioindication. Limnologica. 2013; 164-168; https://doi.org/10.1016/j.limno.2012.08.012.

Klink A, Polechońska L, Cegłowska A, Stankiewicz A. *Typha latifolia* (broadleaf cattail) as bioindicator of different types of pollution in aquatic ecosystems-application of self-organizing feature map (neural network). Environ Sci Pollut Res Int. 2016; 14078-86; https://doi.org/10.1007/s11356-016-6581-9.

Klink A. A comparison of trace metal bioaccumulation and distribution in *Typha latifolia* and *Phragmites australis*: implication for phytoremediation. Environ Sci Pollut Res Int. 2017; 3843-3852; https://doi.org/10.1007/s11356-016-8135-6.

Kumari M, Tripathi BD. Effect of *Phragmites australis* and *Typha latifolia* on biofiltration of heavy metals from secondary treated effluent. Int J Environ Sci Technol. 2015; 1029-1038; https://doi.org/10.1007/s13762-013-0475-x.

Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, Kwon WB, Mathai JK, Navarro DMDL, Jaworski NW, Stein HH. Non-antibiotic feed additives in diets for pigs: A review. Animal Nutrition. 2018; 1-13; https://doi.org/10.1016/j.aninu.2018.01.007.

Lone MI, He Z, Stoffella PJ, Yang X. Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. J Zhejiang Univ Sci. 2008; 210-220.

Lopez-Alonso M, Garcia-Vaquero M, Benedito, JL, Castillo C, Miranda M. Trace mineral status and toxic metal accumulation in extensive and intensive pigs in NW Spain. Livest Sci. 2012; 47-53; https://doi.org/10.1016/j.livsci.2012.02.019.

Lyubenova L, Kuhn AJ, Höltkemeier A, Schröder P. Root exudation pattern of *Typha latifolia* L. plants after copper exposure. Plant Soil. 2013; 187; https://doi.org/10.1007/s11104-013-1634-z.

Lyubenova L, Schröder P. Plants for wastewater treatment - effects of heavy metals on the detoxification system of *Typha latifolia*. Bioresour Technol. 2011; 996-1004; https://doi.org/10.1016/j.biortech.2010.09.072.

Ma Y, Egodawatta P, McGree J, Liu A, Goonetilleke A. Human health risk assessment of heavy metals in urban stormwater. Sci Total Environ. 2016; 557-558: 764-772; https://doi.org/10.1016/j.scitotenv.2016.03.067.

Manios T, Stentiford EI, Millner P. Removal of heavy metals from a metaliferous water solution by *Typha latifolia* plants and sewage sludge compost. Chemosphere. 2003a; 487-494; https://doi.org/10.1016/S0045-6535(03)00537-X.

Manios T, Stentiford EI, Millner P. The effect of heavy metals on the total protein concentration of *Typha latifolia* plants, growing in a substrate containing sewage sludge compost and watered with metaliferus wastewater. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2002;1441-51; https://doi.org/10.1081/ESE-1200013268.

Manios T, Stentiford EI, Millner PA. The effect of heavy metals accumulation on the chlorophyll concentration of *Typha latifolia* plants, growing in a substrate containing sewage sludge compost and watered with metaliferus water. Ecol Eng. 2003b; 65-74; https://doi.org/10.1016/S0925-8574(03)00004-1.

Maric M, Antonijevic M, Alagic S. The investigation of the possibility for using some wild and cultivated plants as hyperaccumulators of heavy metals from contaminated soil. Environ Sci Pollut Res. 2013; 1181-1188; https://doi.org/10.1007/s11356-012-1007-9.

Melià P, Ruggieri G, Sabbadini S, Dotelli G. Environmental impacts of natural and conventional building materials: a case study on earth plasters. J Clean Pro. 2014; 179-186. https://doi.org/10.1016/j.jclepro.2014.05.073.

Milam CD, Bouldin JL, Farris JL, Schulz R, Moore MT, Bennett ER, Cooper CM, Smith Jr S. Evaluating acute effect of methyl parathionapplication in constructed wetland mesocosms. Environ. Toxicol. 2004; 471-479; http://dx.doi.org/10.1002/tox.20052.

Moral R, Moreno-Caselles J, Perez-Murcia MD, Perez-Espinosa A, Rufete B, Paredes C. Characterisation of the organic matter pool in manures. Bioresour Technol. 2005; 153-158; https://doi.org/10.1016/j.biortech.2004.05.003.

Morel JC. Mesbah A. Oggero M. Walker P. Building houses with local materials: means to drastically reduce the environmental impact of construction. Build Environ. 2001; 1119-1126. http://dx.doi.org/10.1016/S0360-1323(00)00054-8.

Mukhopadhyay S, Maiti SK. Phytoremediation of metal enriched mine waste: a review. Global J Environ Res. 2010; 135-150.

Nicholson FA, Smith SR, Allowayc BJ, Carlton-Smithd C, Chambersa BJ. An inventory of heavy metals inputs to agricultural soils in England and Wales. Sci Total Environ. 2003; 205-219; https://doi.org/10.1016/S0048-9697(03)00139-6.

Oves M, Saghir Khan M, Huda Qari A, Nadeen *et al.* Heavy metals: biological importance and detoxification strategies. J Bioremed Biodeg. 2016; 334; https://doi.org/10.4172/2155-6199.1000334.

Peralta JR, Gardea-Torresdey JR, Tiemann KJ, Gomez E, Arteaga S, Rascon E, *et al*. Uptake and effects of five heavy metals on seed germination and plant growth in Alfalfa (*Medicago sativa L.*). B Environ Contam Tox. 2001; 727-734; https://doi.org/10.1007/s001280069.

Peterson H G. Use of constructed wetlands to process agricultural wastewater. Can J Plant Sci. 1998, 199-210; 10.4141/P97-142.

Petroselli A, Giannotti M, Allegrini E, Marras T. Integrated system of phytodepuration for agroindustrial wastewater: Three Different Case Studies. Int J Phytoremediation. 2015; 1227-36; https://doi.org/10.1080/15226514.2015.1045138.

Rafati M, Khorasani N, Moattar F, Shirvany A, Moraghebi F, Hosseinzadeh S. Phytoremediation potential of *Populus Alba* and *Morus alba* for cadmium, chromuim and nickel absorption from polluted soil. Int J Environ Res. 2011; 961-970; https://doi.org/10.22059/ijer.2011.453.

Rai UN, Sinha S, Tripathi RD, Chandra TP. Wastewater treatability potential of some aquatic macrophytes: removal of heavy metals. Ecol Eng. 1995; 5-12; https://doi.org/10.1016/0925-8574(95)00011-7.

Rodriguez-Hernandez MC, García De la-Cruz RF, Leyva E, Navarro-Tovar G. *Typha latifolia* as potential phytoremediator of 2,4-dichlorophenol: Analysis of tolerance, uptake and possible transformation processes. Chemosphere. 2017; 190-198; https://doi.org/10.1016/j.chemosphere.2016.12.043.

Rossi L, Dell'Orto V, Vagni S, Sala V, Reggi S, Baldi A. Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets. Vet Res Commun. 2014b; 39-49; https://doi.org/10.1007/s11259-013-9583-9.

Rossi L, Di Giancamillo A, Reggi S, Domeneghini C, Baldi A, Sala V, *et al*. Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. J Vet Sci. 2013: 263-270; https://doi.org/10.4142/jvs.2013.14.3.263.

Rossi L, Pinotti L, Agazzi A, Dell'Orto V, Baldi A. Plant bioreactors for the antigenic hook-associated flgK protein expression. Ital J Anim Sci. 2014a; 2939; https://doi.org/10.4081/ijas.2014.2939.

Salem ZB, Laffray X, Al-Ashoor A, Ayadi H, Aleya L. Metals and metalloid bioconcentrations in the tissues of *Typha latifolia* grown in the four interconnected ponds of a domestic landfill site. J Environ Sci. 2017. 56-68; https://doi.org/10.1016/j.jes.2015.10.039.

Stroppa N, Onelli E, Hejna M, Rossi L, Gagliardi A, Bini L, Baldi A, Moscatelli A. *Typha latifolia* and *Thelypteris palustris* behavior in a pilot system for the refinement of livestock wastewaters: a case of study. Chemosphere. 2019. 124915; https://doi.org/10.1016/j.chemosphere.2019.124915.

Thangavel P, Subbhuraam C. Phytoextraction: role of hyperaccumulators in metal contaminated soils. Proc Natl Acad Sci. 2004; 109-130.

Todeschini V, Lingua G, D'Agostino G, Carniato F, Roccotiello E, Berta G. Effects of high zinc concentration on poplar leaves: A morphological and biochemical study. Environ Exper Bot. 2011; 50-56; https://doi.org/10.1016/j.envexpbot.2010.10.018.

Venkatarama Reddy BV, Prasanna Kumar P. Embodied energy in cement stabilised rammed earth walls. Energ Buildings. 2010; 380-385; https://doi.org/10.1016/j. enbuild.2009.10.005.

Yadav AK, Abbassi R, Kumar N, Satya S, Sreekrishnan TR, Mishra BK. The removal of heavy metals in wetland microcosms: effects of bed depth, plant species, and metal mobility. Chem. Eng. J. 2012; 501-507; https://doi.org/10.1016/j.cej.2012.09.039.

Yang J, Yez. Metal accumulation and tolerance in wetland plants. Front Biol China. 2009; 282-288; https://doi.org/10.1007/s11515-009-0024-7.

Ye ZH, Baker AJM, Wong MH, Willis AJ. Copper and nickel uptake, accumulation and tolerance in *Typha latifolia* with and without iron plaque on the root surface. New Phytol. 1997;481-488.

CHAPTER 6

General discussion and conclusion

The current situation regarding agricultural activity, intensive food-producing animals and antimicrobial resistance is a significant global concern with a negative impact on human and animal health. Moreover, the first adopted alternative against in-feed antibiotics was the wide application of high doses of zinc and copper salts in the form of premix which, despite their antibacterial and anti-inflammatory activities, raised concerns related to environmental pollution. Furthermore, the use of zinc and copper in feed may also have contributed to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). Therefore, potential source of heavy metal output from livestock wastewater to the environment and increase of antimicrobial resistance have raised many concerns. Therefore, the European Union recently banned the inclusion of pharmacological levels of zinc oxide in animal feed (EMA/394961/2017) and the new maximum admissible level of Cu was also established (EU Regulation, 2018/1039).

In order to reduce the high content of zinc (Zn) and copper (Cu) and decrease the antibiotic use in animal production, plant extracts, different phytochemicals and plant-based strategies are of potential interest due to their antimicrobial, antioxidant and anti-inflammatory properties. This thesis thus focused on integrated approach to reduce input and output of heavy metals and antibiotic use from food-producing animals by (i) overviewing essential nutrients in different farms in Lombardy region, (ii) verifying the *in vivo* effect on different plant based extract such as tannins and leonardite as feed additives on growth performances and blood metabolites of weaned piglets, (iii) testing *in vitro* of pepper mint and spearmint for their anti-inflammatory activity with porcine alveolar macrophages and (iv) evaluating the ability of two aquatic species, *Typha latifolia* and *Thelypteris palustris*, to control the Zn and Cu output from contaminated livestock wastewater.

The overview on the research topic indicated that nutrition plays a pivotal role in the sustainability of the intensive animal rearing system and that, in commercial agricultural industry, heavy metals are represented as both mineral nutrients and contaminant substances. The control of the animal input could be an effective strategy to reduce human health risks related to the spread of resistant bacteria and the environmental pollution by manure even if it represents a valuable source of organic materials for soil fertilization. Using the additives with more precision should be suggested in order to avoid contamination to the environment. Moreover, the results from evaluation of concentration of heavy metals in animal rearing systems experiment showed that undesirable elements (such as arsenic, cadmium, lead, cobalt and nickel) did not exceed the threshold levels thus did not represent any apparent risk for the intensive swine and cattle production systems. The most critical elements in swine farming

were represented by zinc and copper elements probably due to their use in controlling enteric disorders in growing piglets. The main source of zinc and copper in the livestock is represented by feed and the content in the manure reflect the feed content. This study was in line with other worldwide research (Nicholson et al. 1999; Mendoza-Huaitalla et al. 2010; Holzel et al. 2012; Zhang et al. 2012; Wang et al. 2013; Alvarenga et al. 2015; Dai et al. 2016; Li et al. 2018).

An integrated approach is essential not only to promote the sustainability of livestock systems and control the spread of Zn and Cu to the environment but also taking into consideration the profitability of livestock and animal welfare dimensions. In light of this, plants represent an important tool to deal with input and output from agriculture. In the first part we focused on the evaluation of plant-based ingredients to promote gut health of animals. Maintaining animal performance and health by modulating the microbial ecology in the digestive tract and boosting gut health is the most effective strategy for the reduction of antibiotics and consequently of zinc and copper. Plants produce secondary metabolites with functional activities and are therefore, functional feed and food widely studied worldwide (Baydar et al. 2004; Lee et al. 2004; Dundar et al. 2008; Kim et al. 2010; Liu et al. 2012; Huang et al. 2016; Gadde et al. 2017; AlSheikh et al. 2020). Functional feed not only satisfied basic nutritional requirements but also promotes health, improves the immune systems, prevent disease, reduce the risk of chronic diseases and induces physiological benefits beyond traditional feeds. Functional food is as well economically attractive and environmentally friendly. Plants are able to impair the animal gut health through antimicrobial, antioxidant, anti-inflammatory properties thus may be called functional feed and food (Arihara et al. 2014; Lee et al. 2017).

In the first *in vivo* experimental trial, the plant extract tested was quebracho and chestnut tannin, a water-soluble polyphenolic compound able to counteract against pathogens and bacteria. An *in vitro* study by Reggi et al. 2020 showed that balanced combination of tannins at specific dosages may produce a protective and stimulating effect on cell proliferation rather than a cytotoxic effect. Besides, the actual dosage of tannins may be key in determining their effect on bacteria and cells (Reggi et al. 2020). Our results showed that the inclusion of 1.25% (75% of tannins) of quebracho and chestnut tannins did not impair growth performance even if the reduction of protein absorption was observed. Moreover, *in vitro* results on IPEC-J2 cells showed that the presence of tannins in the diet did not impair intestinal cell viability. Furthermore, tannin supplementation modulated serum creatinine and urea concentration. In light of this, we can maximize the positive effect of tannins reducing its dose in diet. In fact, 0.75% of tannins from chestnut and quebracho showed a better zootechnical performance and

faecal scores suggested the beneficial effect of tannins in the prevention of diarrhoea (Caprarulo et al. 2019). In conclusion, different tannins products are available on the market and different studies present heterogeneous compositions of tannin supplementation. Therefore, further studies are needed to find the optimal dosage of tannin inclusion.

In the second *in vivo* experimental trial, leonardite which is a microbial-derived product mainly composed of humic acids derived from the decomposition of organic matter was tested. Albeit, humic acid's own anti-inflammatory and antitoxic proprieties (Islam et al. 2005; Aksu and Bozkurt, 2009) and may provide benefits to piglets' health during post-weaning (Trckova et al. 2017), only limited research with dietary inclusion of leonardite is available. In our experiment where 0.25% leonardite was supplemented, an improvement of the zootechnical performance, serum lipid profile and gut epithelium integrity was found indicating a good general health status of animals. Moreover, the increased serum HDL and decreased total triglycerides suggested that leonardite is a promising feed additive to improve lipid metabolism. In conclusion, leonardite supported an improvement of stress response in weaned piglets thus may be used as an alternative to antibiotics in animal diets.

In the third *in vitro* preliminary data experiment, pepper mint and spearmint were tested *in vitro* for their anti-inflammatory activity with porcine alveolar macrophages as host immune responses. Peppermint and spearmint are known for high content of essential oils (Kalemba and Synowiec 2020) and abundant content of phenolic compounds (Park et al. 2019; Wu et al. 2019). Mint oils as feed additives are therefore being investigated for promoting health in piglets due to their anti-inflammatory, antioxidant and antimicrobial activities (Omonijo et al. 2018). Our *in vitro* experiment revealed that, both mint oils linearly inhibited TNF-α secretion from macrophages with 200 μg/mL as the strongest dose. Results of the current study indicated that both peppermint and spearmint oils had anti-inflammatory activities *in vitro*. Further, *in vivo* animal trials should be conducted to evaluate their impacts on animal health and performance.

In conclusion, tested plant-based additives and phytochemicals showed promising properties and activities and can therefore contribute to reduction of antibiotic and zinc and copper use through the improvement of gut health during the weaning phase. This period exposes piglets to a combination of biological stressors related to physiological, environmental and social challenges (Rossi et al. 2013; Rossi et al. 2014a) and often impairs growth performance, causes fluctuations and dysfunction in gut function and predisposes piglets to digestive disorders and diarrhoea (Moeser et al. 2017; Kim et al. 2019). Thus, in swine production it is crucial to boost pig's gut health by different plant-based extracts and

phytochemicals in the most vulnerable period of swine life cycle. Even if their application results are positive, it is important to think about many factors such as different composition of feed, preparation and mixing of feed, palatability of compounds, legal regulations, cost and benefits of using those compound, which render the plant extracts and phytochemicals applicable in the field and functional in animal industry.

In the first part of my research, I focused on plants as secondary metabolites with bioactive compounds that counteract with pathogens and bacterial infections, which can successfully replace the antibiotics used in animal farming systems and reduce the input of zinc and copper from animal feed. In the second part of my research, I focused on plant-based strategy to reduce output of heavy metals form swine production where plants can be considered as valuable tool for phytoremediation process. This process is simple clean up technology which removes, degradates and immobilizes metals from different matrices such as sludge, soil, sediments, groundwater and wastewater through the use of plants that accumulate large amounts of heavy metals (Bhargava et al. 2012). In light of this, in the fourth outdoor experiment, our results showed that *Typha latifolia* and *Thelypteris palustris* tolerated high levels of Zn and Cu, with no visual toxicity signs and no significant visual effect on their development throughout the experimental period. *Typha latifolia* and *Thelypteris palustris* can thus accumulate and translocate the Zn and Cu from contaminated wastewater. The results suggested that both plant species could be used in a phytoremediation strategy to counteract the output of zinc and copper and possibly other heavy metals from the livestock industry.

I believe that this thesis improves knowledge on the beneficial effects of various plant-based strategies in animal farming and presents valuable knowledge about heavy metals and their critical aspect in animal production. *In vitro, in vivo* and in field studies showed evidence of potential uses of plant extracts and phytochemicals as an alternative to antibiotic use and the Zn and Cu reduction in animal rearing system. My research presented very promising results and the use of novel additives which could be considered as nutritional strategies alternate to input of Zn and Cu and the phytoremediation approach could be validated for green strategy to counteract with high content of contaminants presented in livestock wastewater. To conclude, I strongly opt that plants or plant-based products can be integrated to increase the sustainability of livestock and represent vulnerable sources of active compounds.

AUTHOR'S BIOGRAPHY

Monika was born on August 4th, 1988 in Słupca (Poland). She earned her Bachelor's degree in Biological Sciences (2012) at Adam Mickiewicz University in Poznań (Poland). After the completion of her Bachelor's degree, she enrolled in Adam Mickiewicz University and received her Master's degree (2014) in environmental protection. During her Master program, she conducted research related to ecosystem services in the cities. In 2016, she joined the research group in University of Milan (Italy), Department of Health, Animal Science and Food Safety. One year later she became Ph.D. student under the guidance of Professor Luciana Rossi, with a dissertation mainly focusing on the plant-based strategies to control heavy metals output from swine production. During her Ph.D. program, she led two research projects, made 9 research manuscripts, 12 abstracts with oral or poster presentations, and 4 conferences with 3 oral presentations and 3 poster presentations as a first author and several presentations as co-author.

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