Universitat Autònoma de Barcelona

STRATEGIES OF TRACE MINERAL SUPPLEMENTATION FOR SWINE DEVELOPMENT AND SUSTAINABILITY

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Certify:

That the thesis dissertation entitled "**Strategies of trace mineral supplementation for swine development and sustainability**", presented by Sandra Villagómez Estrada to apply for Doctor degree, has been made under their direction and, considering it finished, authorize its presentation so that it is judged by the corresponding commission.

And for the record to the appropriate effect, sign those present in Bellaterra, September 01, 2021

Dr. José Francisco Pérez Hernández **Dr. David Solà Oriol**

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“Even the smallest things (or doses) can make a big difference”

To my beloved parents,

Yupaychani

Resumen

Durante muchos años, el uso farmacológico de los oligoelementos como el Zn y Cu generó efectos positivos en la industria porcina, al estimular el crecimiento y controlar las disbiosis intestinales, especialmente en los lechones recién destetados. Sin embargo, su excreción excesiva al medio ambiente ha ejercido presión sobre la nutrición animal para encontrar estrategias alternativas en el suministro de estos oligoelementos. El objetivo de esta tesis es explorar los efectos de la reducción de las dosis de minerales traza a través de diferentes fuentes minerales sobre el crecimiento y eficiencia alimentaria animal, así como también en los niveles de excreción fecal y tasas de resistencia a los antimicrobianos, bajo condiciones comerciales.

La reducción de los niveles de suplementación de Zn y Cu a través de fuentes altamente disponibles puede representar una estrategia alternativa para reducir los riesgos de contaminación ambiental. Por lo tanto, en los **Capítulos III** y **V**, se ejecutaron dos experimentos para evaluar los efectos de reducir las dosis de Cu y Zn en el pienso de cerdos destetados y en crecimiento, respectivamente. Los resultados mostraron que la reducción de los niveles de Cu (15 mg/kg) durante seis semanas perjudica el crecimiento de los lechones destetados, con al menos 1 kg de peso corporal, incluso en presencia de la enzima fitasa y particularmente usando la fuente de sulfato. Mientras que la reducción de Zn (20 mg/kg) y Cu (15 mg/kg) a dosis nutricionales en dietas de cerdos de engorde, con enzima fitasa, no afecta el rendimiento ni la composición de la canal, pero sí reduce la excreción fecal de minerales, principalmente con la fuente de hidroxicloruro.

Estimular el consumo temprano de alimento después del destete es de gran interés para la industria porcina. En el **Capítulo IV**, se llevaron a cabo dos experimentos para estudiar la preferencia de los lechones por las dosis y fuentes de Cu. Cuando los lechones pueden elegir entre dietas con niveles nutricionales (15 mg/kg) o niveles razonablemente altos de Cu (150 mg/kg), estos prefieren las dietas con niveles altos, probablemente para restaurar la homeostasis del Cu, y como efecto positivo de la retroalimentación neuroendocrina del Cu sobre el consumo de pienso. En cuanto a las fuentes de Cu, los cerdos prefirieron consumir dietas suplementadas con fuente de hidroxicloruro en lugar de sulfato, posiblemente debido a su sabor menos amargo como consecuencia de sus características de solubilidad.

La gestación de camadas hiperprolificas (> 30 cerdos/cerda/año) genera una presión significativa sobre el rendimiento de la cerda y la camada. En el **Capítulo VI** se evaluó el efecto de la sustitución parcial de fuentes inorgánicas por formas orgánicas, así como una reducción adicional de estas dosis minerales, sobre el desempeño reproductivo de las cerdas y la programación fetal de las camadas. Se detectó por primera vez que la suplementación materna con una combinación de minerales orgánicos e inorgánicos mejora la expresión de varios genes funcionales involucrados en inmunidad (*TGF-β1*, *HSPB1*), barrera (*ZO1*, *MUC2*, *CLDN15*), antioxidante (*GPX2*, *SOD2*), y función digestiva (*CCK*, *IGF1R*), especialmente en los lechones más pequeños de la camada.

En conjunto, la reducción de los niveles de Zn y Cu en las dietas porcinas, principalmente a través de la fuente de hidroxicloruro, disminuye la excreción fecal total y probablemente la presión antimicrobiana sobre las bacterias; no obstante, el rendimiento y el estado fisiológico de los lechones destetados se ve seriamente perjudicado. La nutrición materna con un suplemento parcial de minerales orgánicos es una meritoria estrategia para compensar las desventajas fisiológicas de las camadas hiperprolificas, especialmente en los lechones más pequeños.

Summary

For many years, the pharmacological use of trace elements such as Zn and Cu generated positive effects in the pig industry, by stimulating pig growth and controlling intestinal dysbiosis, especially in weaned piglets. However, their excessive excretion into the environment has put pressure on swine nutrition to find alternative strategies in the supply of trace minerals. The objective of this dissertation is to explore the effects of reducing trace mineral doses through different sources on animal growth and feed efficiency, as well as on fecal excretion levels and antimicrobial resistance rates, under commercial conditions.

Reducing the levels of commercial Zn and Cu supplementation through highly available sources may represent an alternative strategy to reduce the risks of environmental contamination. Therefore, in **Chapters** III and V, two experiments were performed to evaluate reduced levels of Cu and Zn in weaned and growing pigs, respectively. The results showed that reducing Cu levels (15 mg/kg) on post-weaning diets for six weeks impairs the growth of pigs, with at least 1 kg of body weight, even in the presence of the phytase enzyme and particularly with the sulfate source. While the reduction of Zn (20 mg/kg) and Cu (15 mg/kg) at nutritional doses in grower diets, with phytase enzyme, does not affect the performance or the composition of the carcass, but it does reduce the mineral fecal excretion.

Stimulating early feed consumption after weaning is of great interest to the swine industry. In **Chapter** IV, two experiments were conducted to study the pig preference for Cu doses and sources. When pigs are given a choice between nutritional levels (15 mg/kg) or reasonably high levels of Cu (150 mg/kg), they prefer the latter, probably to restore Cu homeostasis, and as a positive effect of the neuroendocrine feedback of Cu

on feed consumption. Regarding Cu sources, pigs preferred to consume diets supplemented with hydroxychloride source instead of sulfate, probably due to the less bitter taste attributed to its chemical solubility differences.

The gestation of hyperprolific litters (>30 pigs/sow/year) generates tremendous pressure on sow and litter performance. In **Chapter VI** the effect of partial substitution of inorganic sources by organic forms, as well as an additional reduction of dietary TM, on reproductive performance and fetal programming of the offspring was evaluated. It was detected for the first time that the supplementation of mothers with a combination of organic and inorganic minerals improves the neonatal expression of several functional genes involved in immune (*TGF-β1*, *HSPB1*), barrier (*ZO1*, *MUC2*, *CLDN15*), antioxidant (*GPX2*, *SOD2*), and digestive (*CCK*, *IGF1R*) function, especially in the smallest piglets of the litter.

Taken together, reducing dietary levels of Zn and Cu, mostly through hydroxychloride source, decreases total fecal excretion and probably pressure on bacteria to develop antimicrobial resistance mechanisms, although performance and physiological state of weaned piglets are seriously affected. Maternal nutrition with a partial supplement of organic minerals is a valuable strategy to counteract the physiological disadvantages on hyperprolific litters, especially in the smallest pigs.

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ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
BW	Body weight
CCK	Cholecystokinin
ermB	Erythromycin resistance gene
EU	European Union
GF	Gain to feed ratio
GHRH	Growth hormone-releasing hormone
GIT	Gastrointestinal tract
GPX	Glutathione peroxidase enzyme
NPY	Neuropeptide Y
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SOD	Superoxide dismutase enzyme
tcrB	Transferable copper resistance gene
tetM	Tetracycline resistance gene
TM	Trace minerals
van	Vancomycin resistance gene

GENERAL INTRODUCTION

For many years, trace minerals (TM) have demonstrated their essentialness that goes beyond physiological requirements and animal wellbeing. Indeed, TM such Zn (>2000 mg/kg) and Cu (>250 mg/kg) are usually provided at extremely high or pharmacological doses to stimulate growth, reduce intestinal dysbiosis and increase feed efficiency, particularly in weanling pigs. This practice gained even more acceptance after the ban of antibiotics as growth promoters in European Union (EU) in 2006. Nevertheless, at these supra doses, TM are not entirely absorbed, resulting in higher quantities of undigested TM excreted in the slurry, which is commonly used as fertilizer for agricultural crops. In such circumstances, in the last years two major concerns (environmental and public health) have put pressure on swine nutrition to find alternative strategies in TM supplementation reducing levels but without affecting growth performance and welfare of animals. The environmental concern considers that the accumulation of Zn and Cu, as heavy metals, in the soil generates greater ecotoxicity in plants, soil microorganisms and water (EFSA FEEDAP, 2016) with the possibility of entering to human and livestock food chain through crops. At the same time, the public health concern ponders that there is an association of these supra levels in swine feed with the increasing microbial resistance rates to antibiotic agents by co-selection within animals, with the additionally possibility of transfer of such resistant bacteria from food-producing animals to humans (i.e. farmers, veterinarians, and consumers) (Yazdankhah et al., 2014; Ciesinski et al., 2018). Consequently, there is a need to improve the administration of TM in feed by exploring different levels, but also different sources that allow less inclusion without affecting its availability for animal performance, while reducing its environmental impact, under commercial conditions.

CHAPTER I

INTRODUCTION

1. Trace minerals as essential nutrients

Intensive pig production demands maximum performance in the different productive periods, for which it is essential to achieve an optimal state of health, especially after weaning. Minerals are inorganic nutrients required by all living organisms for maintenance, growth, and reproduction (Suttle, 2010). Considering the amount in which they are required by animals, minerals are categorized in two groups: macro-minerals and micro-minerals. Macro-minerals are needed in more than 100 mg/kg diet and include Ca, Cl, K, Mg, Na, P, and S elements. Whereas micro-minerals or TM are those required in small quantities (< 100 mg/kg per day), and which are defined also as micronutrients (Suttle, 2010).

Trace minerals are essential factors for carbohydrate, lipid, protein, and vitamin metabolism, as well as for hormone production, immunity, and intestinal function (Suttle, 2010). About twenty elements are defined as TM and are sub-classified, based on their nutritional significance, in three groups: essential elements (Zn, Cu, Mn, Se, Fe, Cr, Co, I, and Mo), probably essential elements (Ni, V, B, Si), and potentially toxic elements (F, Cd, Hg, As, Al, Li, Pb, Sn) (WHO, 1996).

Essential TM such as Zn, Cu and Mn have long been identified as crucial constituents of many enzymes needed for a wide variety of purposes; but in deficit or excess, they could also cause negative impacts on animal physiology (NRC, 2012) and in the environment (EFSA FEEDAP, 2016). The recognition of their essentiality dates to 18th century when it was established that the insufficient supply of minerals caused certain diseases (Suttle, 2010). From here, traditionally, the investigation of the role, deficiency or toxic effects of TM has been performed through trial-and-error experiments

(McDonald et al., 2011), which have enabled to explain their main functions. Nonetheless, the different pathways in which TM perform these functions remain unclear. The development of biotechnological tools, as microbial sequence and gene expression analysis may represent a possible way to further confirm or discard the associated hypotheses to TM mechanisms but also to develop strategies to optimize TM nutrition to pursue a precision nutrition within sustainable production systems.

Though all essential minerals have structural, physiological, catalytic, and regulatory functions (Suttle, 2010), the subsequent sections will briefly summarize all the vital functions of Zn, Cu and Mn in pigs.

1.1. Immune function

A functional immune system is required for the host to have the ability to prevent or respond to pathogen infections. In the body, immune system presents the highest proliferation rate of cells through proliferation and apoptosis process (Kloubert et al., 2018). These processes may become even more important under challenging environments such as an elevate pen density or deficient sanitary situations. In general, Zn, Cu and Mn are continuously involved in cell development and metabolism but, particularly, Zn is widely recognized by playing a central role in immune system (Shankar and Prasad, 1998). Indeed, several animal and human studies show that Zn affects, directly or indirectly, multiple aspects of the immune system, from the skin barrier to lymphocytes gene regulation; and hence any change in Zn dietary level will be perceived by the immune system (Shankar and Prasad, 1998; Skrajnowska and Bobrowska-Korczak, 2019). Copper has also an important role on the innate and acquired immune system and, together with Zn, participates on immune cells differentiation, maturation, and proliferation, as for example in neutrophils, macrophages, and T cell subpopulations (Djoko et al., 2015; Kloubert et al., 2018).

In the pig life, probably the most critical events occur around breeding. Sow nutrition plays a vital role in fetal development and neonate lifetime productivity, including development of immune system (Salmon et al., 2009). However, hyperprolific litters may become an extra challenge for the sow in the path of trying to accomplish neonate competence without neglecting its own (Oliviero et al., 2019). The maternal influence on immune role of Zn was evidenced in broiler breeder progeny (Li et al., 2015). However, studies with TM on sow and its relationship with immune system of litters are scarce. We will try during this thesis to provide new results about the influence of TM in the diets of sow and their effects on their litters. Weaning, per se, is also considered one of the most stressful events in a pig's life with dysfunctions on the immune system associated to diarrhea (Campbell et al., 2013), which might be boosted at early weaning ages (Blecha et al., 1983). The pharmacological Zn (2500 mg/kg) administration to weanling diets have improved the immune system activity (Kloubert et al., 2018), as previously known in humans (Sheikh et al., 2010).

1.2. Antioxidant function

In living organisms, under normal conditions, there is a balance between the formation of free radicals, called reactive oxygen species (ROS), and the antioxidant capacity of radical defense system (Wołonciej et al., 2016). Nevertheless, even small alterations in metabolism or immune response can disturb this delicate balance resulting in an excess of free radicals and/or insufficient protection by antioxidants, leading to a state of oxidative stress (Dröge, 2002; Spears and Weiss, 2008; Wołonciej et al., 2016). Some of the negative effects of oxidative stress are the decreased immune response, lipid peroxidation, DNA, and protein damage and finally cell death (Dröge, 2002; Wołonciej et al., 2016).

Zinc, Cu and Mn are structural components of antioxidant enzymes involved in antioxidant mechanisms, through the group of superoxide dismutase enzymes (SOD) (Cu-Zn-SOD1, Cu-Zn-SOD3, and MnSOD), glutathione peroxidase enzymes (GPX) and ceruloplasmin (Spears and Weiss, 2008; Wołonciej et al., 2016). The SOD enzymes are responsible for dismutation of superoxide radicals to hydrogen peroxide (Spears and Weiss, 2008). Whereas GPX are responsible for neutralizing hydrogen peroxide by reducing it to water and ceruloplasmin, as a Cu transport protein, also exhibits oxidase activity (Spears and Weiss, 2008). Additionally, Zn is also able to induce the synthesis of metallothionein, a metal binding protein that may scavenge hydroxide radicals (Spears and Weiss, 2008; Marreiro et al., 2017). Therefore, under deficient states of these minerals the cellular antioxidant mechanisms can deteriorate. It is worth noting that also in excess or free unbound in bloodstream Cu and Zn could be potentially toxic. When these minerals act as prooxidants they are able to induce the production of intracellular ROS (Bremner, 1998; Gaetke et al., 2014). A commonly used marker to assess lipid peroxidation, and therefore oxidative stress, is the concentration of malonaldehyde in tissues or blood. Several studies confirmed the relationship between elevated Cu and Zn dietary concentrations and a greater oxidative damage in animals. For example, in pigs Cu feed content above to 200 mg/kg increase malonaldehyde levels in duodenum (Fry et al., 2012; Huang et al., 2015) and serum, while decrease GPX, ceruloplasmin and catalase levels in serum (Zhang et al., 2020), and induce hepatic lipid peroxidation (Pu et al., 2016; Zhang et al., 2020). Similar findings were reported by Chen et al., (2020) through an *in vitro* assay with intestinal porcine jejunum epithelial cells under high Zn concentrations (300 µM). It must be considering the potential long-term effect of this imbalanced ROS production on liver function, which may lead to hepatic toxicity and thereby a growth reduction (Floriańczyk, 2003). Interestingly, supplementing pig diets with lower levels of Cu (5 to 120 mg/kg) and Zn (100 mg/kg) have resulted in a gradual increase in serum malondialdehyde but also in an important increase in antioxidant enzymes as GPX, catalase, Cu/Zn-SOD, among others (Liu et al., 2020; Ding et al., 2021). Therefore, exploring a suitable dose of TM to satisfy biological pathways in fast growing animals, as pigs, may improve not only growth but also welfare.

1.3. Reproductive function

An efficiently reproduction system is the base for a successful swine production. Although the role of TM in the reproductive function is often undervalued, they are involved in different components that may, for example, determine litter size, sow productivity and sperm quality (Peters and Mahan, 2008; Suttle, 2010). For instance, Zn is involved in the reorganization of ovarian follicles through the involvement of metalloproteinase-2 enzyme, a member of Zn endopeptidase family (Gottsch et al., 2000). While after farrow, Zn is critical for the repair and maintenance of the uterine lining to return to normal reproductive function and oestrus and with linked improvements in conception rates and embryonic development (Garner et al., 2021). Indirectly, Zn also may improve fertility and maintenance of pregnancy by reducing locomotor incidence problems such as lameness and osteochondrosis (Fabà et al., 2018). Copper is also involved in steroidogenic enzymes cytochrome P450, 17αhydroxylase and cytochrome P450 side-chain cleavage and lysyl oxidase (Kendall et al., 2006). In dairy cattle, adequate serum Cu levels are associated with less days for first service, fewer services per conception and fewer days to open (Harvey et al., 2021). Whereas Mn participates in the synthesis of the steroids, estrogen, progesterone, and testosterone (Harvey et al., 2021). In females with regular reproductive cycles the concentration of Mn in vagina has been found in higher levels than in anoestrous animals (Hidiroglou, 1979).

In reproductive mammals as age progresses all these TM functions acquires further importance and sows are no exception. As sow parity increases there is a gradual depletion on their mineral reserves restricting sows to maintain a high level of productivity (Mahan and Newton, 1995), and which may be more critical in modern hyper prolific lines. Indeed, Mahan and Newton, (1995) showed that sows from 3rd parity had a 15-20% lower concentration of TM than non-reproductive sows of the same age, suggesting that the daily supply of TM must be increased with the sow's age. Moreover, is common that old sows (> 6th parity) have a greater number of pigs born dead per litter compared to younger sows, probably attributed by an increase in farrowing duration associated to a loss on uterine muscle tone for poor mineral reserves or oxytocin secretion (Langendijk and Plush, 2019). Another factor that influences the farrowing length, and hence the incidence of stillborn piglets, is the size of litter, being critical as litter size increases (Vanderhaeghe et al., 2013). In both cases, younger or older sows with small or large litters, a proper supply of macro and micro-minerals is considered important for the effective contractions of muscles surrounding the uterus, improving the transport of fetuses through the birth canal (NRC, 2012; Theil, 2015). In that sense, Vallet et al., (2014) showed that supplementing late gestating sows with greater levels of Zn (700 mg/kg) than those recommended (100 mg/kg; NRC, 2012) decreased stillbirth rate, probably by decreasing birth intervals. Interestingly, lower levels of Zn (72 to 595 mg/kg), Cu (15 to 30 mg/kg) and Mn (20 to 50 mg/kg) in gestating sows did not affect the number of stillborn, born alive or total born piglets (Holen et al., 2020; Ma et al., 2020; Tsai et al., 2020).

An additional critical point in swine reproduction is the undeniable challenge that the increase in prolificity has generated to swine producers. Nowadays, it is increasingly common for commercial farms to manage hyperprolific maternal lines with the objective of obtaining a greater number of pigs born and weaned per sow and year (>30 pigs), thus improving their efficiency and economic return. Nutritionally, this means an increase in the nutritional demand of sows to maintain their own health, productive longevity, and efficiency while ensuring the growth and viability of their offspring. In highly prolific lines as the number of fetus increase the average pig birth weight is reduced (even lower than 800 g), which instead is associated with a greater risk of pre-weaning mortality (Kemp et al., 2018). In general, the presence of small piglets (weighing less than 1 kg) within litters may increase from 7 % in small size litters (11 piglets) to 25 % in larger litters (≥ 16 piglets) (De Vos et al., 2014; Feldpausch et al., 2019), with up to 30 % of these piglets exposed to different degrees of intrauterine growth restriction (Foxcroft et al., 2006; Amdi et al., 2013). Some nutritional interventions during pregnancy, such as the supplementation of diets with functional amino acids (Mateo et al., 2007; Nuntapaitoon et al., 2018) have been proposed to mitigate the incidence of small piglets. <u>Since TM have structural and metabolic functions (Suttle, 2010) it is possible that an improved TM supplementation will also improve the neonate viability</u>. Recently, Holen et al., (2020) suggested that a higher Zn supplementation (365 and 595 mg/kg), than requirements, during late gestation enhanced pig birth weight and reduced the incidence of low-birth weight pigs compared to a lower supplementation (125 mg/kg). To underline this relationship, maternal nutrition not only plays a critical role in fetal growth and development but also is the major factor that alters expression of the fetal genome and hence may have lifelong consequences on the offspring (Wu et al., 2004; Ji et al., 2017). In poultry breeders the in-feed Zn supplementation (70 vs 20 mg/kg) positively regulated the expression of proinflammatory and intestinal barrier function genes in progeny (Li et al., 2015). In swine nutrition, consistent information is scarce and very little research has focused on the perinatal transfer of maternal nutrients, as minerals, and their effects on the offspring programming.

1.4. Growth promoter function: intestinal microbial modulation

All over the world, the commercial swine practice usually includes elevated doses of TM, mainly Zn and Cu, to improve pig growth performance and feed efficiency (Figure 1). However, exact modes of action by why these TM, low or elevated doses, improve animal performance are not completely known.

One of the major suggested hypotheses is that high inclusion levels of Zn (> 2000 mg/kg) and Cu (170-300 mg/kg) promote growth through their antimicrobial effect in the animal gastrointestinal tract (GIT), in a similar way to antibiotic-based growth promoters, controlling and reducing the invasion and adhesion of pathogens (Højberg et al., 2005; Yu et al., 2017; Zhang et al., 2019).

Mechanisms of antibacterial activity of Zn and Cu ions are not complete known but some proposed activities include: a) membrane damage caused by direct or electrostatic interaction between the ion and microbial cell surfaces; b) cellular internalization of ion nanoparticles; and c) the production of ROS in microbial cells due to metal oxides (Pasquet et al., 2014; Sirelkhatim et al., 2015). The last seems to be the strongest antimicrobial mechanism, since the induction of ROS inactivate cell components, such as nucleic acids, lipids, and proteins resulting immediately in bacterial death (Djoko et al., 2015). Zinc and Cu antimicrobial properties have been tested in both *in-vitro* (Pang and Applegate, 2007; Almoudi et al., 2018) and *in-vivo* assays through plate counting (Højberg et al., 2005; Namkung et al., 2006; Mei et al., 2009; Pieper et al., 2012) and ultimately by using high-throughput sequencing analysis (Pieper et al., 2020), which provide a more comprehensive understanding of microbial communities.

However, Zn and Cu effect is not lethal for all intestinal bacteria species, from the literature is stated that some bacterial groups, mostly pathogens, are suppressed whereas others increase (Broom et al., 2021). Overall, the supplementation of Zn (>2000 mg/kg) and Cu (> 175 mg /kg) at pharmacological doses have resulted in the suppression of some commensal and pathogenic bacteria (e.g., *Clostridium*, *Salmonella*, coliform populations), frequently involved in post-weaning diarrhea, with a few increases in beneficial populations (e.g. *Lactobacillus, Bifidobacterium*) (Højberg et al., 2005; Song et al., 2013; Jensen, 2016; Pieper et al., 2020; Broom et al., 2021). Interestingly, when Zn (100-200 mg/kg) and Cu (100-150 mg/kg) are supplemented in moderate or lower doses, the intestinal microbiota profile continues to be affected, although with less evident modifications than with pharmacological ones (Mei et al., 2009; Jensen, 2016; Zhang et al., 2019; Pieper et al., 2020). Since mineral sources, by which Zn and Cu are supplemented, have different solubility and chemical characteristics within GIT, they may affect the intestinal microbiota differently. Consequently, it is worthy to evaluate doses and sources in which Zn and Cu are still effective in controlling microbial pathogens while minimizing their negative collateral effects.

Besides of this bacterial modulation, the pharmacological content of Zn and Cu in pigs might also exert a positive effect, directly or indirectly, on intestinal morphology and function, which results into an improvement in pig efficiency (Stensland et al., 2015; Dębski, 2016; Hung et al., 2020; Pieper et al., 2020). The bacterial modulations of Zn and Cu toward the reduction of opportunistic pathogens together with the development of saprophytic bacteria may lead to a significant reduction on bacterial toxic metabolites such as ammonia (Pieper et al., 2012; Pieper et al., 2020) and biogenic amines (Song et al., 2013), hence increasing nutrient amounts destined for growth performance. On the other hand, many of these saprophyte microbial populations are also able to produce short-chain fatty acids (SCFA; i.e., butyrate, propionate, and acetate) (Tungland, 2018), which instead are essential forms of energy in host intestine (Ríos-Covián et al., 2016). This last could also be associated with improvements on intestinal morphology such as low crypt depth and great villous length when Cu was provided at 200 mg/kg to pigs (Zhao et al., 2007) and Zn at 2500 mg/kg (Li et al., 2001).

Moreover, as mentioned before, Zn is particularly essential for normal intestinal barrier function and the regeneration of damaged gut epithelium; therefore, an increased Zn concentration (160 to 2500 mg/kg) in the intestine may also influence on host local defense mechanisms such as mucin composition (P. Liu et al., 2014), and expression of intestinal genes related to innate immunity and inflammatory processes (*ZO-1*, *OCLD*, *IL-1β*, *IFN-γ*, *TGF-β*) in young pigs (P. Liu et al., 2014; Pearce et al., 2015; Zhu et al., 2017).

Undoubtedly, the dynamic relationship between the pharmacological dose of TM, microbiome, and the host has successful helped weanling pigs to overcome post-weaning challenges (environmental and nutritional), especially in the exposition to several pathogens that lead into acute diarrheas. Nonetheless, it should not be lost sight the harmful collateral effects on microbial resistance rates to Zn and Cu but also to antibiotic agents by co-selection of these pharmacological doses (Yazdankhah et al., 2014; Ciesinski et al., 2018). Which have engendered increasing legislative pressure on swine nutrition aimed to get a more accurate supply of trace minerals. For instance, in the last years, European regulations have limited the inclusion levels of Zn and Cu in the feed for different pig productive phases (European Comission, 2016; European Commission, 2018) in addition to ban the medicinal use of Zn as zinc oxide at pharmacological doses and which will be completely withdrawn in 2022 (European Commission, 2017). Consequently, the swine industry needs to find environmentally sustainable nutritional strategies (together with management practices) that allow it to achieve production objectives within legislative limits. These important concerns will be reviewed further in the coming sections.

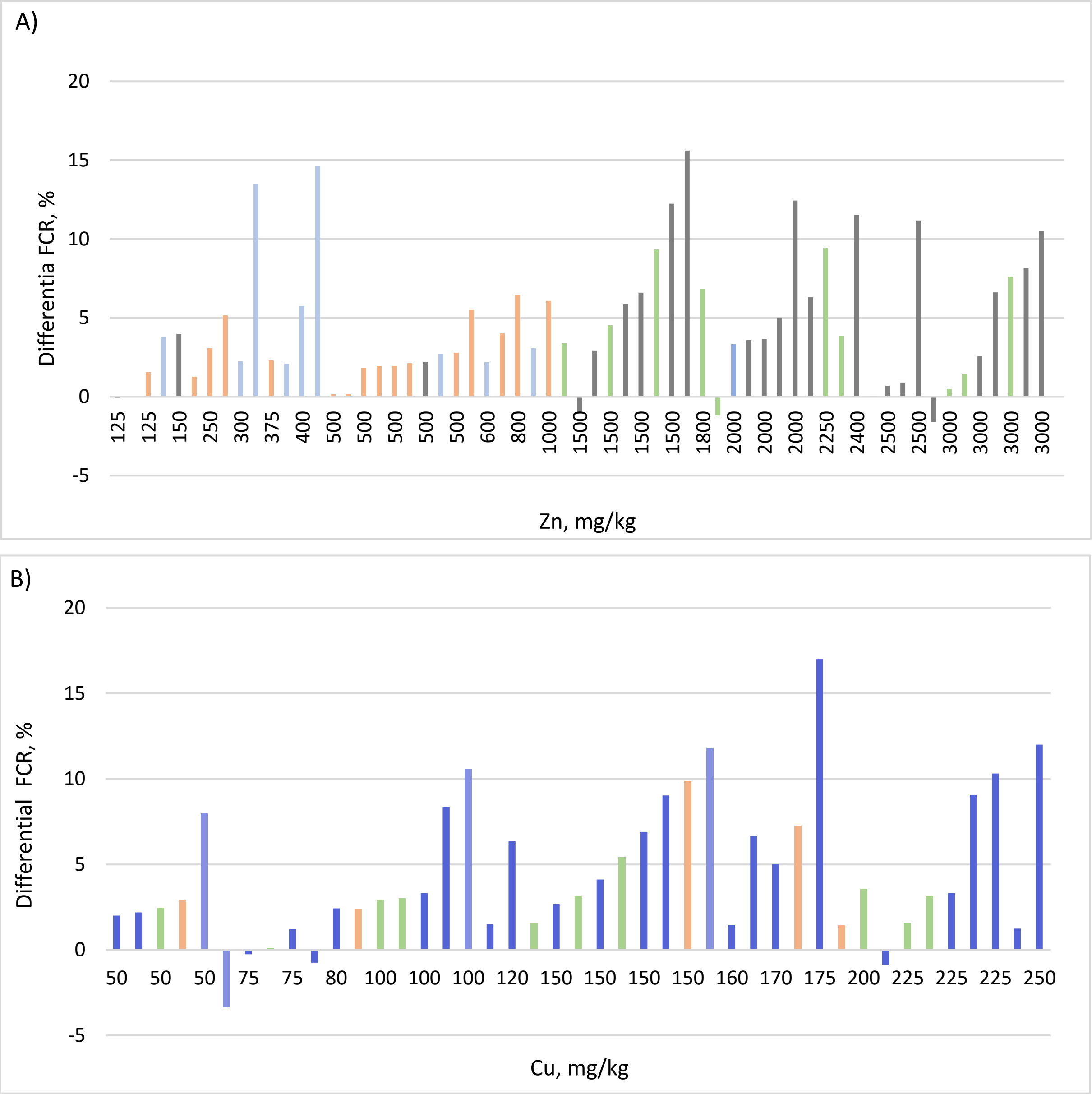

Figure 1. Feed conversion rate of weaned pigs expressed as differential percentage between control group (plain or nutritional dose) and the correspondent supplemented Zn (A) and Cu (B) high dose group. Source legend: ●Oxide, ● Organic, ●Hydroxychloride, ● Sulfate, ● modified Zn oxide sources (nano and coat), ● modified Cu sulfate sources (nano and coat). The studies utilized to create this graph are listed in appendix.

1.5. Growth promoter function: feed intake and metabolism activity

In 1994, Zhou et al. showed that also an intravenous injection of Cu to weaned pigs, similar to 200-250 mg Cu/kg in feed, was able to stimulate their growth, thus sharpening the hypothesis that not only the antimicrobial effects of GIT are behind the mechanisms of Cu action but also a systemic effect. Since then, it has been reported that Cu supplementation may regulate some growth, feeding and fasting signaling pathways, improving protein deposition and promote bone formation (Gao et al., 2020). Indeed, multiple studies with pigs reported an increased feed consumption with high Cu levels (150-250 mg/kg) (Hill et al., 2000; Bikker et al., 2015; van Kuijk et al., 2019; Blavi et al., 2021). The mechanism of growing function has been related to the release of neuropeptides (Yang et al., 2011). Particularly, the growth hormone-releasing hormone (GHRH) and somatostatin, together with other nutrient-specific signals such as neuropeptide Y (NPY), noradrenaline, galanin, and orexin may determine the circadian expression of food intake in animals (Yang et al., 2011). Since Cu could be transported through the blood-brain barrier, as a free Cu ion, it could participate in regulation of neuroendocrine action in the brain (Choi and Zheng, 2009) and therefore in its related neuropeptides. Indeed, high dietary Cu levels (175 and 250 mg/kg) seems to stimulate appetite in pigs by upregulating NPY mRNA expression and enhancing NPY concentration in the hypothalamus (Li et al., 2008; Zhu et al., 2011) along with the reduction on mRNA abundances of anorexigenic proopiomelanocortin in hypothalamus (Zhu et al., 2011). Another metabolic pathway considers the increased of GHRH mRNA levels which instead increases the growth hormone mRNA expression in the pituitaries of pigs as response to high Cu levels (125 mg/kg) (Yang et al., 2011). Additionally, Gonzalez-Esquerra et al., (2019) demonstrated that addition of 150 mg Cu/kg increased the mRNA expression of ghrelin, which is often called as hunger hormone due to its role in regulating feed intake and body weight (BW) on pigs.

Regarding the role of Zn, it is widely accepted that one of the clinical signs of its deficiency in pigs is the profound anorexia and interruption of growth (Blaabjerg and Poulsen, 2017), but which might be rapidly reversed through a Zn sufficient diet. Nevertheless, the connection between the Zn deficit and loss of appetite is uncertain. Some suggested hypotheses are that Zn stimulates feed intake through orexigenic peptides coupled to the afferent vagus nerve (Suzuki et al., 2011), by increasing ghrelin secretion (Yin et al., 2009) and increasing insulin-like growth factor expression in the small intestine mucosa (Li et al., 2006). An increased levels expression of NPY and orexin mRNA were observed in rats after an orally Zn administration in response of a short-term Zn deprivation (Suzuki et al., 2011).

Besides the feed intake effects, Zn and Cu may also impact certain aspects of lipid metabolism and enzymatic activity (Dove and Haydon, 1992; Dove, 1993; Luo and Dove, 1996; Suttle, 2010). Indeed, the inclusion of Cu in rabbit diets (45 mg/kg) and in pig diets (150 mg/kg) improved growth performance by upregulating mRNA genes involved in uptake, transport, metabolism, and oxidation of fatty acids (Espinosa et al., 2017; Lei et al., 2017); indicating the role of Cu in signaling routes of lipid post absorptive metabolism. Moreover, Cu seems also to influence protein synthesis. In a broiler study, the supplementation of Cu at 100 mg/kg besides to improve growth performance, immunological capacity and modulation of intestinal microbiota increased serum total protein content and albumin concentration while reducing the level of urea nitrogen compared to no addition of Cu (Wang et al., 2011). Likewise, the supplementation of 175 or 250 mg Cu/kg to weaned pig diets improved the digestibility of crude protein and ether extract (Mei et al., 2009). Regarding Zn, in deprived animals a low plasma and liver concentrations of liposoluble vitamins E and vitamin A were observed (Suttle, 2010) suggesting an important relationship between Zn and vitamin metabolism. In quails, the Zn supplementation at 60 mg/kg moderately alleviate the effects of heat stress, in part, by secondary improvements in vitamin status, along with increases in feed intake, egg production and egg quality (Sahin and Kucuk, 2003).

2. Deficiency and toxicity

As reviewed in the previous paragraphs and based on all the essential functions of Zn, Cu and Mn, it is not surprising that deprived animals develop critical dysfunctions on growth, reproduction, and immune function. Indeed, under Zn deficiency pigs experiment a systemic failure of metabolism which results in growth impairment, decreased water and feed intake, parakeratosis, and other subclinical signs (Tucker and Salmon, 1955; Brugger and Windisch, 2019). Among these, parakeratosis is the pathognomonic clinical sign and may mark the end point in response to a long-term insufficient supply of Zn (Brugger and Windisch, 2016). Whereas a Cu dietary deficiency state may be related to hematological (anemia) and neurological (paraparesis and ataxia in the hind limbs) manifestations accompanied with reduction in growth performance and feed intake (Suttle, 2010; Olinda et al., 2017; Wazir and Ghobrial, 2017). In the reproductive system, deficiency of Zn, Cu, or Mn results in suppression of estrus, retained placenta, embryonic death, and decreased conception rates (Kellogg et al., 2004; Nocek et al., 2006).

Undoubtedly, all these classic signs caused by Zn, Cu and Mn deficiency are of important concern, but perhaps the one with the greatest physiological and productive repercussion is the alteration that their deficiency causes in the immune response of animals and thereby in growth performance, particularly in sanitary challenging conditions. Zinc and Cu deficiency, especially, is known to reduce the chemotactic response and, therefore, the phagocytic capacity and bactericidal activity of the organism towards pathogens (Djoko et al., 2015; Kloubert et al., 2018), thus leaving the animal unprotected, which would add much more challenge to overcome the post-weaning syndrome in young pigs and to optimize health and production in fattening pigs and breeding sows.

By contrast, an excess of these TM can also cause physiological problems, with reduced growth rate being the common indicator of mineral toxicity. This toxicity is usually reached after a prolonged feeding at pharmacological doses. Even though pigs are more tolerant to Cu toxicosis than ruminants (Goff, 2018), prolonged feeding of Cu (300-500 mg/kg) will mainly cause liver damage, since it is the main storage of Cu after its absorption (Gaetke et al., 2014). The pathognomonic clinical sign of this toxicosis is jaundice (NRC, 2012). Liver is not only an important organ for metabolism but is also part of the frontline on immune defense, therefore a healthy liver is directly related to healthy pigs. Furthermore, excessive Cu supply in pigs can lead to the opposite effect of increased feed intake reviewed in the previous section. Gao et al., (2020) extensively reviewed the consequences of prolonged Cu feeding in pigs, which include oxidative damage of some nerve cells weakening the feeding signal transmission, and other as chronic gastritis and ulcers, reduction on some digestive enzymes activities, reduction on the rate of digestion, prolong chyme transit time in the intestine, enhance satiety and thus inhibit food intake.

Toxic effects of Zn on pigs may include arthritis, gastritis, bone, and cartilage deformities (Straw et al., 2006). The supply of Zn in pharmacological doses (> 2000 mg/kg) during the first two weeks after weaning has been a useful tool in the control of post-weaning diarrhea; however, longer supplementation has resulted in the opposite effect by reducing feed intake and growth rate with metabolic disorders (Martin et al., 2013; Pieper et al., 2015). While for Mn chronic toxicity the major reported lesion is Fe deficiency, resulting from an inhibitory effect of Mn on Fe absorption. Additional signs can include depressed growth, anorexia, and altered brain function (Keen and Zidenberg-Cherr, 2003).

Overall, it must be emphasized that a pharmacological Cu (300 mg/kg) and Zn (2500 mg/kg) supply in young pig diets represents about 60 and 30 times higher than their requirements (5-6 mg Cu/kg; 80-100 mg/Zn kg; NRC, 2012), respectively. Therefore, it would be reasonable that after a long-term overdose the homeostasis mechanisms can overcome causing toxicity, reduced growth, and ultimately death; without considering the counteracted effects on the metabolism of other minerals and the phytase efficacy (Walk et al., 2015). An important issue to contemplate in mineral toxicity may be the chemical nature of TM, since it appears that the administration of highly absorbable sources over a prolonged period increases the risk of toxicity (NRC, 2012).

3. Requirements and recommendations

The term “nutritional requirement” refers to the minimum amount of certain nutrient needed to meet the requirements for maintenance, well growth, production, and reproduction in all living organisms. Compared to amino acids or energy, very little research has been focused on TM nutrition on swine area. Usually, the macronutrient requirements as amino acids, N, Ca, and P, are generated by a modelling approach; contrary to the requirements for TM which are derived from a critical evaluation of nutrient deficiency studies (NRC, 2012). Therefore, requirements for TM are mostly considered as the minimum level required to overcome a deficiency condition rather than to boost productivity or immunity. Most of these studies were conducted 40-50 years ago and since the 1988 edition of the NRC they have not changed, except for breeding sows, whose quantities have increased (NRC, 2012, Table 1). It is worth noting that under stressful conditions such as the first days after weaning, or even more critical when pigs are weaned at very young ages, the innate feed intake of pigs is low or non-existent, hence supply these low nutritional levels would not be appropriate to satisfy the pig physiological needs.

In commercial swine production, there are also references providing the amount of TM given as “recommendations”. Usually, nutritional recommendations are values given for a population, where there are different categories of pigs (e.g., heavy, and light weight), and therefore the requirements are focused for an average pig within the population or herd. These safety margins usually are considered as the amount necessary for bodily function beyond maintenance (Table 1). Nevertheless, it must be considered that these requirements and recommended values will differ according the sanitary and immunological status of pigs. For instance, the pig Zn requirement may be increased under stressing or poor sanitary conditions to optimize the immune function (Klasing, 2001).

Table 1. Dietary trace mineral requirements (mg/kg feed) and recommendations for pigs allowed feed ad *libitum*

Productive phase	Requirements[1]			Recommendations[2]				
	NRC (1988)	NRC (1998)	NRC (2012)	INRA (1984)	BSAS (2003)	FEDNA (2013)	Rostagno (2011)	Rostagno (2017)
	United States	United States	United States	France	United Kingdom	Spain	Brazil	Brazil
Zn, mg/kg								
Young pigs, 5-25 kg	80.0 - 100.0	80.0 - 100.0	80.0 - 100.0	100.0	100.0	100.0 - 130.0	110.0 - 123.0	113.0-156.0
Growing- finishing pigs, 25-135 kg	50.0 - 60.0	50.0 - 60.0	50.0 - 60.0	100.0	100.0	90.0 - 120.0	55.0 - 88.0	53.0-92.0
Sow gestating	50.0	50.0	100.0	100.0	80.0	95.0 - 120.0	110.0	120.0
Sow lactating	50.0	50.0	100.0	100.0	80.0	95.0 - 120.0	110.0	120.0
Cu, mg/kg								
Young pigs, 5-25 kg	5.0 - 6.0	5.0 - 6.0	5.0 - 6.0	10.0	6.0	8.0 - 15.0	12.0 - 13.0	13.0-17.0
Growing- finishing pigs, 25-135 kg	3.0 - 4.0	3.0 - 4.0	3.0 - 4.0	10.0	6.0	8.0 - 13.0	6.0 - 10.0	6.0-10.0
Sow gestating	5.0	5.0	5.0	10.0	6.0	10.0 - 15.0	12.0	13.0
Sow lactating	5.0	5.0	5.0	10.0	6.0	10.0 - 15.0	12.0	13.0
Mn, mg/kg								
Young pigs, 5-25 kg	3.0 - 4.0	3.0 - 4.0	3.0 - 4.0	40.0	30.0	35.0 - 60.0	40.0 - 45.0	42.0-57.0
Growing- finishing pigs, 25-135 kg	2.0	2.0	2.0	40.0	30.0	18.0 - 45.0	20.0 - 32.0	20.0-34.0
Sow gestating	10.0	20.0	25.0	40.0	20.0	40.0 - 50.0	40.0	44.0
Sow lactating	10.0	20.0	25.0	40.0	20.0	40.0 - 50.0	40.0	44.0

Diets provided under thermoneutral and optimal sanitary conditions.

[1] NRC trace mineral requirements are given for several BW ranges. The present values are expressed as a range, corresponding the first value to heavier pigs and the second value for smaller pigs. [2]FEDNA, (2013), consider young pig requirements in a range of BW between 5 to 20 kg and growing-finishing pigs from 20 to more than 100 kg. Rostagno recommendations consider inorganic TM supplementation for young pig requirements in a range of BW between 5 to 30 kg and growing-finishing pigs from 30 to 125 kg. BSAS (2003), consider young pig requirements in a range of BW between 10 to 30 kg and growing-finishing pigs from 30 to 60 kg.

4. Current supplementation: levels and sources

Since the TM content in raw ingredients is low, variable (due to its agricultural process and phytate content) or not complete known, the dietary TM supplementation rely exclusively on the vitamin-mineral premix composition. Usually, the TM supplementation on sow diets follow the recommended levels, but it does not occur the same with nursery and grower-finisher diets, which tend to exceed 1.6 to 18.7 and 23.3 to 25.8 times the NRC values for Zn and Cu, respectively (Flohr et al., 2016), on account of its growth promoting effects. However, due to the negative impacts associated with pharmacological doses of TM (reviewed in the subsequent paragraphs) it is necessary to re-evaluate the commercially available sources to reduce and optimize mineral supplementation.

Commercially, several TM sources are available to be used in the swine diets. These compounds are often chosen according to their solubility characteristics, mineral content percentage, relative bioavailability, and economic value; and they are usually identified as inorganic or organic sources (Table 2). Generally, to estimate the relative bioavailability of a mineral source, it is compared to a known standard which is usually the sulfate source and through a dose-response studies that evaluate the change of suitable variable responses. For instance, the relative bioavailability of Cu from Cu carbonate source expressed as an availability relative to Cu sulfate range between 60 to 100 %, which means that compared to 100% of bioavailability of Cu sulfate at least 60 % of Cu from carbonate may be available to be used by the animal. It must be noted that the relative bioavailability does not refer to the percentage of mineral absorbed or retained by the animal; the absorbed and retained mineral as a percentage of intake is usually less than 50% of the intake (NRC, 2012). This true bioavailability may be defined as the proportion of an ingested nutrient that is absorbed, transported to its site of action, and be utilized to the physiologically active species (O'Dell, 1983). Although it might be thought that water-soluble sources may be easily absorbable and therefore more available for animal physiology process, there are several doubts about their actual efficacy that have emerged from published animal studies comparing different mineral sources and according to Jerry W. Spears, (1996) organic and inorganic forms are metabolized differently following absorption.

However, evaluating the Zn, Cu or Mn sources bioavailability in animals is not an easy mission. The special features of TM metabolism, including a variable endogenous intestinal excretion, a rapid turnover of the mineral in blood and a constant urinary and fecal excretion coupled with a wide range of dietary intakes, limit the methods for measuring the real mineral absorption (Suttle, 2010). Typically, in estimating the relative bioavailability of different TM sources, slope-ratio assays have been used (Littell et al., 1995), where the different responses of the animals are indicative of their TM status. Responses may include serum or plasma mineral content, metalloproteins content in serum or liver, tissue mineral content (e.g. liver, bone, gallbladder), fecal digestibility, enzyme activities, and ultimately the novel gene expression analysis.

Table 2. Common Zn, Cu and Mn source characteristics

Mineral element	Source	Compound	Chemical formula	Element content, %	Relative bioavailability, %
Zn	Inorganic	Oxide	ZnO	72	50-80
		Sulfate monohydrate	$ZnSO_4.H_2O$	35.5	100
		Sulfate heptahydrate	$ZnSO_4.7H_2O$	22.3	100
		Carbonate	$ZnCO_3$	56	100
		Chloride	$ZnCl_2$	48	100
	Hydroxychloride	Tetrabasic chloride	$Zn_5(OH)_8Cl_2\cdot H_2O$	55	111
	Organic	Amino acid complex	Variable	Variable (<26)	-

		Proteinate	Variable	Variable (10-15)	100
Cu	Inorganic	Sulfate pentahydrate	$CuSO_4.5H_2O$	25.2	100
		Oxide	Cu_2O	89	100
			CuO	80	0-10
		Carbonate	$CuCO_3$	50-55	60-100
	Hydroxychloride	Tribasic chloride	$Cu_2(OH)_3Cl$	54	112
	Organic	Polysaccharide complex	Variable	Variable (<26)	90-124
		Proteinate	Variable	Variable (10-15)	105-111
Mn	Inorganic	Sulfate monohydrate	$MnSO_4.H_2O$	29.5	100
		Carbonate	$MnCO_3$	46.4	30-100
		Chloride	$MnCl_2$	27.5	100
		Oxide	MnO	60	70
		Dioxide	MnO_2	63.1	35-95
	Organic	Polysaccharide complex	Variable	Variable (<26)	120-125
		Proteinate	Variable	Variable (10-15)	110

Adapted from Ammerman et al., (1995); Baker, (2000); NRC, (2012)

In general, the inorganic sources include oxides, sulfates, chlorides, and carbonates compounds and they are characterized to be ionically bonded to an inorganic salt. Oxides were traditionally used in weaning diets, frequently associated with Zn. However, given that its positive effect requires preeminent inclusion levels (> 2000 mg/kg), in addition to its effects associated with environmental pollution and its recent ban in EU, it must be replaced by other more available sources that need less inclusion doses. Currently, as non-medicinal products, sulfates may be the most widely used sources in commercial swine nutrition. They are recognized for their relatively low cost and for being highly soluble in water and acid solutions compared to organic and hydroxychloride sources (Guo et al., 2001; Park and Kim, 2016). Nevertheless, this solubility characteristic also makes them very prone to interactions with other components of the diet, including phytic acid and other minerals (Santos et al., 2015; Ren et al., 2021), which may interfere with their absorption and hence availability. As a partial solution to these undesirable characteristics of inorganic sources, technological processes have been proposed such protecting the sulfate and oxide molecule by a lipid coat (Pastorelli et al., 2014; Upadhaya et al., 2018) or reducing the particle size of oxides (nanoparticles; < 100 nm) (Pei et al., 2019). Though these novel proposals need deeper evaluation to show their benefits in animal health and production.

The other TM category is the organic sources, or as habitually referred to as “chelates”. In their chemical structure the soluble metal salt is covalently bonded to either protein, peptide, amino acid, or organic acid molecule (AAFCO, 2002). This “protected” structure makes them more stable and less reactive with feed ingredients in the digestive tract (Acda and Chae, 2002) and is possible they may be absorbed more efficiently than sulfates (Y. Liu et al., 2014). However, its high price limits its inclusion, especially when large quantities of feed are used for fattening or reproduction; therefore, a valuable strategy may be partial replacement with cheaper inorganic sources but without compromising the TM availability within the animal. Early studies with young and growing pigs have shown promising results (Veum et al., 2004; Hill et al., 2014; Zhao et al., 2014; Liu et al., 2016). However, little is known about the effects of these strategies on the performance of gestating sows (Ma et al., 2020; Tsai et al., 2020) and, in particular, their likely effects on fetal programming of litters born from hyperprolific sows.

An intermediate type of TM source is hydroxychloride. They are characterized by a crystalline structure formed by covalent bonds located between the soluble metal ion, multiple hydroxyl groups and the chloride ions (Zhang and Guo, 2007). This covalent bond structure clearly differs from the ionic bond of common inorganic sources (e.g. sulfates) but is similar to the covalent bonds present in organic sources (Figure 2). The difference is that in organic sources the metal ion is covalently bound to a carbon-containing ligand, while in the hydroxychloride minerals the ion is covalently bound to a hydroxyl groups and chloride ions (Arthington, 2015). Based on this characteristic, hydroxychloride minerals have low solubility in water but high solubility in acid solutions (Cao et al., 2000), which make them less reactive with other components in the diet, such as vitamin E and phytase activity, compared to the highly soluble sulfates (Luo et al., 2005; Lu et al., 2010). Additionally, Arthington (2015) highlight the non-hydroscopic and free dust value that hydroxychloride minerals have, which may be an advantage during feed manufacture, as well as their highly element concentrated which takes up less space within feed formulation compared to other less concentrated sources.

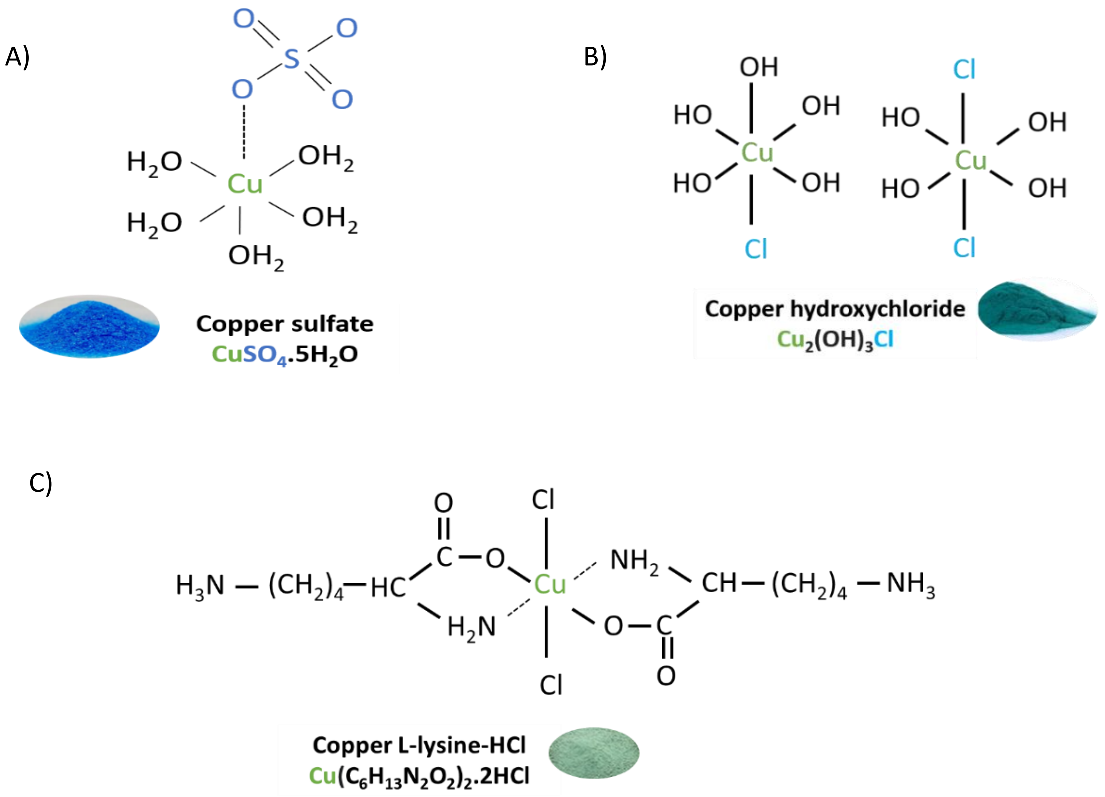

Figure 2. Chemical structure of Cu sulfate (A), Cu hydroxychloride (B) and Cu L-Lysine-HCl sources (C). Adapted from EFSA FEEDAP, (2013) and Selko IntelliBond C, Trouw nutrition manufacturer.

An important factor to consider within the nutrition of TM is the role of the phytase enzyme as a useful tool to improve the availability of minerals not only the innate content from cereals but also from the digestion of minerals added in the premix. It is well known that phytic acid, the principal storage form of P in cereals, acts as the major binding molecule of nutrients in diets, and which complexation with cations reduced the bioavailability of Zn, Mn and, in lesser extent, Cu in pig diets (Schlegel et al., 2010; Bikker et al., 2012). Several studies have shown the efficacy of phytase in improving TM availability thus representing an affordable approach to reduce the dietary inclusion of these nutrients, especially of Zn (Schlegel et al., 2010; Bikker et al., 2012). However, the ease of bonding between the phytate and the mineral element may vary depending on the nature of the mineral source, for example inorganic sources such as Zn oxide and Cu sulfate are more prone to chelation (Pang and Applegate, 2006; Santos et al., 2015). In addition, these same complexes can interfere with the efficacy of phytase, as has been seen with Cu sulfate, impairing the release of P and other minerals from the phytate molecule (Banks et al., 2004; Pang and Applegate, 2006; Hamdi et al., 2018).

5. Environmental and public health consequences and European Legislation

The absorption and excretion of Zn and Cu are strictly regulated through efficient homoeostatic control mechanisms, and when these minerals are supplemented over the animals' requirements, the excess is not absorbed or is endogenously secreted, mainly to intestinal pathways (pancreatic and bile secretions) (J. W. Spears, 1996; Goff, 2018), ending up in manure. As continuously pointed out during this revision, for many years, the swine industry has supplemented diets with pharmacological doses of Zn and Cu, that besides to affect the availability of other nutrients within digestive tract of animal (Pang and Applegate, 2006; Pang and Applegate, 2007; Walk et al., 2015), also generate a large quantity of these undigested elements in manure. For instance, the Cu supplementation at 250 mg/kg ($CuSO_4$) caused 12 times increase of fecal Cu excretion in young pigs compared a diet at 10 mg/kg ($CuSO_4$) (2 831 vs 232 mg Cu/kg) (Armstrong et al., 2004). The subsequent use of this manure as a fertilizer in the soil may be a severe threat to environment and public health (EFSA FEEDAP, 2016).

Heavy metals as Zn and Cu are prone to accumulate in soil increasing the ecotoxicity of plants, microorganisms, soil, and ground water (Dębski, 2016; EFSA FEEDAP, 2016). As explained by Wuana and Okieimen, (2011) soils contaminated with these elements may cause direct threats on crop growth and yield, and indirect threats by entering the human food chain with a potentially negative impact on human health. Moreover, the toxicity of these elements may negatively influence the activity of soil microorganisms and earthworms, thus retarding the breakdown of organic matter (Wuana and Okieimen, 2011).

Added to this, there is also an increasing public health concern due to the association of the great dietary, and therefore environmental, Zn and Cu contents with the increasing microbial resistance rates to antibiotic agents by co-selection, as well as the possibility of transferring of such resistant bacteria from food-producing animals to humans such as farmers, veterinarians, and consumers (Yazdankhah et al., 2014; Ciesinski et al., 2018). To protect themselves, from the toxic effects of Zn and Cu, bacteria evolved a range of mechanisms (Grass et al., 2011; Yazdankhah et al., 2014; Djoko et al., 2015). The possible connection between Cu-Zn resistance and antibiotic resistance is based on two mechanisms: cross-resistance (physiologically), and co-resistance (genetically) (Seiler and Berendonk, 2012). Co-resistance refers to the presence of two or more genetically linked resistance genes usually located next to each other on one mobile genetic element as plasmids (Seiler and Berendonk, 2012). Thus, Hasman and Aarestrup, (2002) found that Cu resistance genes are often located on transferable plasmids (*tcrB*) and which were identified in several *Enterococcus* species (Hasman and Aarestrup, 2002; Hasman et al., 2006). Interestingly, the same plasmid was also found to carry genes *ermB* and *vanA*, which encode resistance to macrolides and glycopeptides, respectively (Hasman and Aarestrup, 2002; Hasman et al., 2006). Nowadays glycopeptide antibiotics, such as vancomycin, belong to the group of last alternative antibiotics in human medicine.

In such circumstances and after several risk-benefit analyzes, the European Commission concluded that the benefit of using high doses in animal feed does not outweigh the risks to the environment. Therefore, the current maximum allowable levels of Zn and Cu for swine feed in EU countries are described in table 3, although a further reduction is not excluded (EFSA FEEDAP, 2014). A similar situation occurred with the use of pharmacological levels of Zn as oxide source, whose use as a veterinary medicinal product in post-weaning diets has been banned in the EU countries (European Commission, 2017).

Table 3. Current maximum levels of Zn and Cu in the diet (mg / kg feed) for pigs allowed as feed ad *libitum*

Productive phase	EU allowance, mg/kg feed	
	Zn	Cu[1]
Young pigs, 5-25 kg	150	150-100
Growing- finishing pigs, 25-135 kg	120	25
Sows	150	25

European Commission (2016) and European Commission (2018)

[1] EFSA regulation allow the Cu supplementation of 150 mg/kg for pigs up to 4 weeks post-weaning followed by a reduction to 100 mg/kg until 8 weeks after weaning (European Commission, 2018).

6. Justification of the project

For several years the pharmacological or elevated doses of trace minerals such as Zn and Cu have successfully helped swine industry to overcome critical periods in pig life, such as weaning, by increasing pig feed intake, controlling intestinal dysbiosis or improving nutrient utilization. Nevertheless, there are also some negative collateral effects associated with these supra-doses in the environment and public health that demands reducing TM supplemented levels in food-producing animals. Since there are different sources of minerals in commercial practice, with different attributes, exploring strategies to supplement Zn, Cu and Mn at reduced levels through single or combined sources represents a great approach to optimize TM nutrition in the swine industry.

In this scenario, four studies will be developed to test the following hypotheses:

i. Reducing the commercial supplementation levels of Zn and Cu through highly available sources can represent an alternative strategy to diminish the risks of environmental contamination without impairing the productive performance of post-weaning and fattening pigs.

ii. Moderately high levels of Cu in post-weaning diets can be a beneficial nutritional approach to stimulate weanling pig appetite and therefore promote early feed consumption while exerting a positive microbial modulation in the gastro-intestinal tract.

iii. Feeding gestating sows with functional doses and sources of trace minerals might impact positively on neonatal development and programming of pigs born from large litters, particularly in the smallest pigs.

CHAPTER II

OBJECTIVES

The present dissertation aims to explore the effects of reduced trace mineral supplementation through different sources on animal growth performance and feed efficiency, as strategies to reduce public health and environmental impact.

To achieve this objective, four specific objectives, reflected in four experimental works, are established:

1. To evaluate in newly weaned pigs the effect of two Cu dietary reduced levels as well as the effect of two Cu and Zn sources on growth performance, tissue mineral status, microbial modulation, and presence of antimicrobial-resistant genes.
2. To investigate the feed preference of weaned pigs by Cu sources and doses; and to determine the TM properties in similar conditions to those found in the oral cavity and digestive tract by testing *in vitro* the Cu solubility of each source and its interaction with phytic acid.
3. To assess in grower-finisher pigs the effect of two Zn and Cu sources, supplemented at lower levels than those established by the EU regulations, on growth performance, carcass characteristics, tissue mineral content and mineral apparent total tract digestibility. To further understand the solubility characteristics of the Zn sources, through an *in vitro* study, as well as the degree of interaction with phytic acid, as the main dietary binding molecule.
4. To determine the effect of partial substitution of inorganic sources for organic forms, as well as an additional reduction of dietary TM on the reproductive performance and mineral status of hyperprolific sows (>30 piglets/ sow/year). To understand the influence of maternal nutrition on the fetal programming of

the offspring and their mineral status, through the evaluation of two different categories of newborn pigs (light and average BW)

CHAPTER III

Effects of copper and zinc sources and inclusion levels of copper on weanling pig performance and intestinal microbiota

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NON RUMINANT NUTRITION

Effects of copper and zinc sources and inclusion levels of copper on weanling pig performance and intestinal microbiota

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Abstract

A 42-d experiment was conducted to evaluate the effect of Cu and Zn source and Cu level on pig performance, mineral status, bacterial modulation, and the presence of antimicrobial-resistant genes in isolates of *Enterococcus* spp. At weaning, 528 pigs (5.9 ± 0.50 kg) were allotted to 48 pens of a randomized complete block design in a 2 × 2 factorial arrangement with two Cu and Zn sources (**SF**: sulfate and **HCl**: hydroxychloride) and two Cu levels (15 and 160 mg/kg). As a challenge, the pigs were reared in dirty pens used by a previous commercial batch. Two-phase diets were offered: the pre-starter (**PS**) phase from day 1 to 14 and the starter phase (**ST**) from day 14 to 42. At days 14 and 42, pigs were individually weighed and blood samples from one pig per pen were taken. At the end of the experiment, one pig per pen was euthanized to collect the samples. Feeding high levels of Cu increased body weight (**BW**) from 16.6 to 17.7 kg ($P < 0.001$). Furthermore, average daily gain, gain to feed (**G:F**) ratio, average daily feed intake (**ADFI**), and mineral status were enhanced with Cu at 160 mg/kg ($P < 0.05$) compared with Cu at 15 mg/kg. There was no effect of the interaction between source × level on any of the growth performance responses except for ADFI ($P = 0.004$) and G:F ($P = 0.029$) at the end of the ST period and for G:F ($P = 0.006$) for entire nursery period (day 0 to 42). At the end of the ST period, pigs fed Cu at 160 mg/kg as HCl had not only higher ADFI but also lower G:F than those fed Cu as SF at 160 mg/kg. Meanwhile, for the entire nursery period, G:F did not differ between pigs fed Cu at 160 mg/kg as HCl or SF. In colonic digesta, the relative abundance of *Streptococcus*, *Enterobacter*, *Escherichia*, among others, decreased (P-adjust < 0.05), while *Lachnospira* and *Roseburia* tended (P-adjust < 0.10) to increase in pigs fed Cu at 160 mg/kg as HCl compared with those fed Cu SF at 160 mg/kg. An increase (P-adjust < 0.05) in *Methanosphaera* and *Roseburia* was observed in pigs fed Cu at 160 mg/kg. From colon digesta, *Enterococcus* spp. was isolated in 40 samples, being *E. faecalis* the most dominating (65%) regardless of the experimental diet. Genes of *ermB* (7.5%) and *tetM* (5%) were identified. No genes for Cu (*tcrB*) or vancomycin (*vanA*, *vanB*, *vanC1*, and *vanC2*) were detected. In conclusion, European Union permissible levels of Cu (160 mg/kg), of both sources, were able to increase performance, mineral status, and bacterial modulation compared with nutritional level. Different effects on growth performance, mineral tissue content, and microbial modulation were observed between Cu and Zn sources.

Key words: antimicrobial resistance genes, copper, European levels, microbiota, weaned pigs, zinc

Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
AMP	ampicillin
BW	body weight
CLIN	clindamycin
CLOR	chloramphenicol
CP	ciprofloxacin
DNA	deoxyribonucleic acid
ENR	enrofloxacin
ERY	erythromycin
EU	European Union
FDR	false discovery rate
G: F	gain to feed
GEN	gentamicin
GIT	gastrointestinal tract
GPX	glutathione peroxidase
HCl	hydroxychloride
ICP-OES	inductively coupled, plasma-optical emission spectroscopy
IMI	imipenem
KAN	kanamycin
MIC	minimum inhibitory concentration
NDF	Neutral detergent fiber
NMDS	nonmetric dimensional scaling
PCR	polymerase chain reaction
PG	penicillin G
PRRS	porcine reproductive and respiratory syndrome virus
PS	pre-starter period
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCFA	short-chain fatty acids
SEM	standard error of the mean
SF	sulfate
SOD	superoxide dismutase
ST	starter period
ST	streptomycin
TBCC	tribasic copper chloride
TET	tetracycline
VAN	vancomycin

Introduction

Copper and zinc are essential trace minerals involved in many physiological processes (Olivares and Uauy, 1996). The National Research Council (**NRC**) established a minimum requirement of Cu (6 mg/kg) and Zn (100 mg/kg) for weanling pigs (NRC, 2012). However, stressing factors at weaning frequently result in low feed intake, gastrointestinal disorders, and, consequently, impaired gut integrity and growth (Lallès et al., 2007). In the European Union (**EU**) until 2003, and today in other regions of the world (including the United States), commercial practice generally used high doses of Cu (200 to 250 mg/kg) and Zn (2,000 to 3,000 mg/kg) as therapeutic additives in post-weaning diets. The main positive effects attributed to these therapeutic doses are growth promotion (Cromwell et al., 1998) and antimicrobial activity (Højberg et al., 2005; Namkung et al., 2006). However, at high dietary levels, Cu and Zn are barely absorbed in the intestine, affecting the availability of other nutrients (Pang and Applegate, 2006, 2007) and generating a major environmental concern (European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed; EFSA FEEDAP, 2016) as well as a public health risk due to microbial tolerance and resistance to other antimicrobial agents (Hasman and Aarestrup, 2002; Van Noten et al., 2016). Based on these considerations, the EU approved new maximum levels of Cu for pigs, being 150 mg/kg up to 4 wk after weaning, followed by a reduction to 100 mg/kg until 8 wk after weaning (European Commission, 2018). The previous regulation allowed the inclusion of Cu at 170 mg/kg in diets up to 12 wk of age, when this experiment was performed. The current total feed content of Zn for pigs until 11 kg body weight (**BW**) is 150 mg/kg (European Commission, 2016); however, a further reduction to 110 mg/kg is not excluded (EFSA FEEDAP, 2014).

Sulfate (**SF**) is the most used mineral source in swine diets. It is characterized by a labile molecular bond that allows high solubility in water and acid solutions and is commonly used as a reference to compare the bioavailability of different mineral sources (Park and Kim, 2016). Alternatively, hydroxychloride (**HCl**) mineral sources have a crystalline structure formed by covalent bonds, with slow solubility in the gastrointestinal tract (**GIT**) and a high amount of biologically active ions (Cohen and Steward, 2014). Thus, in the present study, we hypothesize that due to differences in chemical properties of trace mineral sources, growth performance of early weaned pigs fed diets supplemented with HCl mineral sources will be higher or similar than those fed SF, hence offering an alternative to the use of high levels of SF and antimicrobials as growth promoters. The low solubility of HCl trace minerals, which are different from SFs, makes them less prone to antagonistic nutrient interactions at the proximal section of GIT, hence increasing mineral and nutrients availability as well as probably promoting a greater impact on intestinal microbiota. Thus, the objective of this study was to compare the effect of two sources of Cu and Zn (SF and HCl) as well as the effect of two Cu dietary levels (15 mg/kg as nutritional or 160 mg/kg as high level) on growth performance, mineral status, microbial modulation, and the possible presence of antimicrobial-resistant genes in newly weaned pigs. Pigs were allocated to dirty pens used by a previous nursery batch in order to provide a more challenging scenario due to early contact with a dirty and non-disinfected environment.

Materials and Methods

All animal experimentation procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

Animals and housing

The experiment was performed on a commercial farm in Catalonia, Spain. At weaning (21 d), 528 pigs ([Large White × Landrace] × Pietrain) obtained from the same commercial farm and with an initial average BW of 5.9 ± 0.50 kg were used in a 42-d study in a commercial nursery unit. Pigs were ear tag identified, blocked according to the initial BW, and distributed into four experimental diets in 48 pens (12 pens per treatment, 11 pigs per pen). Entire males and females were randomly assigned to the same pen. The pigs were housed in dirty pens used by a previous commercial batch in order to increase the environmental challenge. Each pen (3.12 m^2) had a fully slatted floor and was equipped with a commercial non-lidded hopper (TR5, Rotecna, Spain) and a nipple drinker to provide ad libitum access to feed and water. The facility was environmentally controlled (temperature and ventilation rate) by the use of thermostatically controlled heaters and exhaust fans depending on the age of the pigs (28 to 22 °C). Pigs were allotted to two identical rooms. Each room had 28 pens divided by a central feeding corridor but only 24 pens were used in the experiment (the two at the far ends of the room close to the doors were discarded and used as refusal/hospital pens). In order for the weaned pigs to be kept in poor sanitary conditions, the pens were not cleaned or disinfected after use by a previous commercial batch. Since the pens had a fully slatted floor, an excessive amount of feces was not accumulated. Ventilation and temperature were adjusted prior to the housing of the newly weaned pigs. The commercial swine farm is stable but positive for the porcine reproductive and respiratory syndrome (**PRRS**) virus. The standard farm practices include the vaccination of pigs at 20 d of age against porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (Suvaxyn Circo + MH RTU, Pfizer, Spain) and the vaccination of sows against PRRS (MSD, Spain) every 4 mo. Pigs are weaned at 21 d and with an average BW of 5.8 kg. Nursery period is 6 wk with a daily weight gain ranging between 280 and 290 g and 2% to 3% mortality. Zinc oxide is added to feed at pharmacological levels (2,500 mg/kg) for 1 wk. The usual inclusion of Cu to weaned pig diets is 9 mg/kg. Antibiotics are administered after veterinary prescription if required to treat specific diseases.

Experimental design and dietary treatments

Two-phase diets (Table 1) were formulated to meet or exceed nutrient requirements (NRC, 2012): the pre-starter (**PS**) phase from day 1 to 14 and the starter (**ST**) from day 14 to 42.

Four experimental diets were prepared in a 2 × 2 factorial arrangement, with two Cu and Zn sources (SF and HCl) and two Cu inclusion levels (nutritional: 15 mg/kg and high: 160 mg/kg). Supplementation of Zn was fixed for all diets at 110 mg/kg. Cu SF pentahydrate (25%) and Zn SF monohydrate (35%) were obtained from Pintaluba, Reus, Spain. The HCl Cu (54%, IntelliBond C) and Zn (55%, IntelliBond Z) were obtained from Trouw Nutrition, the Netherlands. A vitamin–mineral premix without Cu and Zn was prepared. For each dietary treatment, Cu and Zn products were premixed with 25 kg of basal diet before being put directly in the mixer during the feed preparation process. In order to avoid cross contamination with elements from previous production, feed was prepared in an appropriate rank order starting with the lower concentrations to be included in the diet. The first and last 100 kg of the final pellet diet from each batch (experimental treatment) were discarded to reduce cross contamination. All diets were offered ad libitum in pellet form. Composite samples (1 kg) were collected during the bagging process in representation of each experimental treatment. Each sample was, therefore, proportionally split into four 250 g samples that were stored for further analysis. Zinc oxide was not added at pharmacological levels in the diets and no antibiotics or feed additives with antimicrobial properties were used.

Table 1. Composition of the basal diets for the two phases, as-fed basis[1]

Ingredients, %	Pre-starter	Starter
Wheat	26.32	40.60
Maize	6.75	20.00
Barley	12.20	15.38
Soybean meal 47% CP	0.90	12.98
Fishmeal	4.00	5.00
Lard	2.58	2.48
Soybean meal heat treated	3.60	—
Extruded wheat	13.05	—
Porcine plasma	3.00	—
Dextrose	4.00	—
Acid milk whey	4.50	—
Sweet milk whey	8.50	—
Extruded soybeans	7.15	—
Di calcium phosphate	1.36	1.40
Calcium carbonate	0.18	0.11
L-Lysine 50	0.80	0.85
L-Threonine	0.22	0.24
DL-Methionine	0.25	0.16
L-Tryptophan	0.02	0.05
Salt	0.22	0.35
Vitamin premix nucleous[2]	0.40	0.40
Calculated composition		
DM	90.0	89.1
NE, kcal/kg	2,550	2,401
CP	19.5	17.9
NDF	10.8	10.3
Ether extract	6.5	4.8
Ca	0.70	0.60
Total P	0.68	0.68
Dig P	0.40	0.40
Analyzed composition		
DM	91.5	90.2
CP	18.7	18.6
Ether extract	6.3	6.3
NDF	7.8	7.8
Ash	5.1	4.8

[1] Pre-starter phase diets were fed from day 0 to 14 and starter phase diets were fed from day 14 to 42.

[2] Provided per kg of feed: vitamin A (acetate): 12,000 IU; vitamin A (retinol): 2,000 IU; vitamin D3 (cholecalciferol): 1,204 IU; vitamin D (25-hydroxicholecalciferol): 600 IU; vitamin E: 104 IU; vitamin K3: 2 mg; vitamin B1: 3 mg; vitamin B2: 7 mg; vitamin B6: 3.5 mg; vitamin B12: 0,1 mg; D-pantothenic acid: 17 mg; niacin:45 mg; biotin: 0.2 mg; folacin: 1.5 mg; Fe (chelate of amino acid): 15 mg; Mn (oxide): 6.25 mg; Mn (chelate of glycine): 3.75 mg; I (calcium anhydrous): 1.75 mg; Se (organic): 25 mg; Se (sodium): 50 mg. Phytase: 1,500 FYT (Ronozyme NP (M), DSM, Basel, Switzerland).

Experimental procedures and sampling

The BW of each pig and feed left in the feeders were recorded on days 14 and 42. From these data, average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed (**G:F**) ratio were calculated. At the end of the PS phase, one pig per pen was selected based on the mean BW within the pen (median) to take samples of blood by jugular puncture. Samples from the same animal were taken at the end of the ST phase. For antioxidant enzyme determination, blood was collected into 4 mL vacutainer tubes containing lithium heparin (BD Vacutainer, LH, BD-Plymouth, UK) and centrifuged at 3,000 × *g* for 15 min. The acquired plasma was stored at −80 °C for further analysis. Meanwhile, blood samples for Cu and Zn determination were collected into 5 mL vacutainer tubes free of detectable Zn. Serum was obtained after centrifugation (3,000 × *g* for 15 min) and immediately frozen at −20 °C. At the end of the experimental period, the selected pig per pen (n = 12) was euthanized with an overdose of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Madrid, Spain); organ samples (liver and left tibia) were collected to determine Cu and Zn concentrations. Finally, digesta from the proximal colon (1 m from the ileocecal junction) were collected for microbiota analyses and detection of antimicrobial-resistant genes. Samples were immediately stored at −20 °C until processing and analysis.

Chemical analysis

Analytical determinations of diets were performed according to the AOAC International (2005) methods for dry matter (method 934.01), crude protein with the Dumas Method (method 968.06), ether extract was determined using traditional Soxhlet extraction (method 920.39), and ash (method 942.05). Neutral detergent fiber was analyzed using the Ankom nylon bag technique (Ankom 200 fiber Analyzer, Ankom Technology, Macedon, NY).

The activities of superoxide dismutase (**SOD**) and glutathione peroxidase (**GPX**) in plasma were determined by spectrometry and following the instructions of Ransod and Ransel kits, respectively (Randox, County Antrim, UK). Liver was dried in a forced-air oven at 102 °C per 12 h and then milled at 0.5 mm. Tibia was autoclaved to remove all the adjacent muscle and tissue (121 °C for 30 min). Subsequently, tibia was oven-dried for 12 h at 102 °C and soaked in acetone under a chemical hood for 48 h to extract fat. After this period, tibia was again oven-dried for 12 h at 102 °C and then broken in the middle before being ashed overnight at 550 °C in a muffle furnace. Samples of feed were milled at 0.5 mm before mineral analysis. All samples were solubilized in nitric acid prior to mineral analysis by inductively coupled, plasma-optical emission spectroscopy (**ICP-OES**, model Optima 4300DV, PerkinElmer, Inc.; Waltham, MA, US).

Microbial molecular analysis

Bacterial DNA was extracted from 200 mg of colonic digesta by using the commercial MagMAX CORE Nucleic Acid Purification 500RXN Kit (Thermo Fisher, TX, US) and following the manufacturer's instructions. For 16S rRNA gene sequence-based analysis, the V3–V4 regions of the bacteria 16S ribosomal RNA gene were amplified by polymerase chain reaction (PCR; 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and 72 °C for 5 min) using primers F5′-barcode- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and R5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. A negative control of the DNA extraction was included as well as a positive Mock Community control to ensure quality control. After 25 cycles of amplifications, 550 pb amplicons were obtained. The Illumina Miseq sequencing 300 × 2 approach was used. Raw sequencing reads were quality clipped, assembled, and compared with available genomic sequences using a Microomics Systems S.L (Barcelona, Spain) software and were validated and subsequently completed with the Kraken Metagenomics (Wood and Salzberg, 2014) and QIIME (Caporaso et al., 2010) software. Taxonomic assignment of phylotypes was performed using a Bayesian classifier trained with Silva database version 132 (99% Operational taxonomic units full-length sequences) (Wang et al., 2007).

Enterococcus spp. isolation, detection of resistance genes, and phenotypical antimicrobial resistance tests

Colon digesta samples were plated and incubated on Slanetz-Bartley agar (Oxoid, UK) for 48 h at 37 °C. Colonies morphologically compatible with *Enterococcus* spp. were confirmed by PCR (Dutka-Malen et al., 1995). In parallel, using a boiling method (Queipo-Ortuño et al., 2008), bacterial DNA was extracted from Slanetz–Bartley agar plates to detect the following antimicrobial resistance genes: Cu (*tcrB*) (Hasman et al., 2006), vancomycin (**VAN**; *vanA*, *vanB*, *vanC1*, *vanC2*) (Dutka-Malen et al., 1995; Kariyama et al., 2000), tetracycline (**TET**; *tetM*), and erythromycin (**ERY**; *ermB*) (Jacob et al., 2008). In vitro susceptibility of *Enterococcus* spp. isolates was determined by the disk diffusion method using 13 antimicrobial agents: VAN (30 µg, BD, US), penicillin G (**PG**; 10 µg, Oxoid, UK), ampicillin (**AMP**; 25 µg, BD, US), imipenem (**IMI**, 10 µg, BD, US), ERY (15 µg, BD, US), TET (30 µg, BD, US), ciprofloxacin (**CP**; 5 µg, BD, US), enrofloxacin (**ENR**; 5 µg, BD, US), clindamycin (**CLIN**; 2 µg, BD, US), gentamicin (**GEN**; 10 µg, BD, US), kanamycin (**KAN**; 30 µg, BD, US), streptomycin (**ST**; 10 µg, BD, US), and chloramphenicol (**CLOR**; 30 µg, BD, US). The Clinical and Laboratory Standards Institute cutoff values were used. Additionally, a minimum inhibitory concentration (**MIC**) test was performed to assess the susceptibility of *Enterococcus* spp. to Cu SF pentahydrate using the broth microdilution method. Isolates were cultured for 24 h in wells plates with brain heart infusion broth supplemented with 0.5, 1, 2.5, 5, 10, 15, and 20 mM of Cu SF pentahydrate. The ATCC 29212 *Enterococcus faecalis* strain was used as a quality control.

Statistical analysis

Data were analyzed as a randomized complete block design using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC, US). The model included the fixed effects of source, level source × level interaction, and the random effects of block. Pen was the experimental unit for performance response. Mineral concentration in organs, antioxidant measurements, and microbiota community were analyzed with an individual pig as the experimental unit. The normality and homogeneity of the data were examined using the Shapiro-Wilk test. The concentration of Cu in the liver exhibited heterogeneity; therefore, it was log-transformed before analysis. Serum mineral content was analyzed as repeated measures. Biostatistical analyses were performed in open source software R Studio v.3.5.1. Diversity was analyzed at the species level using a vegan package (Oksanen et al., 2017). Richness and alpha diversity were calculated with raw counts based on Shannon and Inverse Simpson estimators. Betadiversity was evaluated by multivariate ANOVA based on dissimilarities with the adonis function. To compare any differential effects from treatments, an ANOVA was performed for richness and diversity. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log-normal mixture model; *P*-values were corrected by false-discovery rate (**FDR**) with the metagenomeSeq package (Paulson et al., 2019).

Due to factorial arrangement, the main effects are discussed for responses in which the interaction was not significant. Significantly different means were separated using Tukey adjust. Significance was declared at a probability $P \leq 0.05$ and tendencies were considered when the *P*-value was between > 0.05 and < 0.10.

Results

Analyzed mineral concentrations in feed were according to those planned. In SF diets, Cu level was 9.5 and 107.7 mg/kg for nutritional and high Cu level diets, respectively, whereas, Zn content was 79.8 and 162.9 mg/kg for nutritional and high Cu SF diets, respectively. Likewise, in HCl diets, the Cu content for nutritional (9.9 mg/kg) and high (133.4 mg/kg) diets was according to that expected. The Zn content was 110.1 and 176.1 mg/kg for nutritional and high Cu HCl diets, respectively. The difference between low and high levels of Cu in the diets was achieved with both SF and HCl Cu sources.

Growth performance

Growth performance response for the interaction between two sources of Cu and Zn and two Cu levels is shown in Table 2. Feeding diets with the higher Cu level increased the ADFI, BW, and ADG during the PS and ST periods, being the final BW increased from 16.6 to 17.7 kg ($P = 0.0002$). Likewise, G:F increased as Cu inclusion in the diet increased ($P = 0.038$), but no effects of Cu and Zn source were observed on growth performance ($P > 0.10$). There was no effect of the interaction between source × level on any of the growth performance responses except for ADFI ($P = 0.004$) and G:F ($P = 0.029$) at the end of the ST period and for G:F ($P = 0.006$) for the entire nursery period (day 0 to 42). At the end of the ST period, pigs fed Cu at 160 mg/kg as HCl had not only a higher ADFI but also lower G:F than those fed Cu SF at 160 mg/kg. For the entire nursery period, G:F did not differ between pigs fed Cu at 160 mg/kg as HCl or SF. Mortality was 2.46% and was not related to any dietary treatment ($P > 0.10$).

Antioxidant activity

The activity of GPX on pigs fed Cu at nutritional level was greater than that for those fed Cu at a high level (3,389 vs. 3,004 U/L; $P = 0.013$) at the end of the ST period. The GPX activity tended to be higher in animals fed Cu and Zn HCl than those fed Cu and Zn SF (3,437 vs. 3,144 U/L; $P = 0.057$) at the end of the PS period. No interactions between source × level on the activity of SOD or GPX in plasma was observed ($P > 0.10$; Table 3).

Mineral content in organs and tissues

Feeding diets with higher levels of Cu increased the liver and serum content of Cu and Zn ($P < 0.05$; Table 4). Pigs fed Cu and Zn HCl had a greater Cu content in the liver ($P = 0.036$) and serum ($P = 0.037$) than those fed Cu and Zn SF. No effect of mineral source on liver and serum Zn concentrations was observed ($P > 0.10$). There was no effect of the interaction between source × level on Cu and Zn content on liver and serum except for Zn content on the tibia ($P = 0.044$). Pigs fed Cu HCl at high level had a greater accumulation of Zn (277.3 mg/kg) than those fed high Cu level as SF (256.1 mg/kg). All treatments had low levels (< 0.02 mg/g) of Cu storage in bone, below the ICP-OES detection limit.

Microbial molecular analysis

A two-way interaction between source and level was observed for alpha estimators (Table 5). The Shannon and Inverse Simpson indexes were lower in pigs fed a high Cu level as HCl compared with those fed Cu SF ($P < 0.05$). Beta diversity analysis revealed distances between clustered samples of nutritional and high Cu level group ($P_{ADONIS} = 0.001$) and a tendency for the two-way interaction between source and level ($P_{ADONIS} = 0.054$; Figure 1a and b).

At the family level, 224 different families were identified. From those, the families most frequently reported to change are presented in Figures 2 and 3. Diets with high Cu levels increased the relative abundance of Chrysiogenaceae, Halomonadaceae, and Ruminococcaceae and decreased the abundance of the Acetobacteraceae and Brucellae families (P-adjust < 0.05; Figure 2). Regarding Cu and Zn source effect, Vibrionaceae family decreased (P-adjust = 0.027) and Methylobacteriaceae tended to increase more in pigs fed HCl minerals than in those fed SF minerals (P-adjust < 0.10; Figure 3).

At the genus level, 554 different genera were detected. From those, the genera most frequently reported to change are presented in Figures 2 and 3. Pigs fed Cu at 160 mg/kg had a higher relative abundance of *Methanosphaera* and *Roseburia* genera compared with those fed nutritional levels (P-adjust < 0.05; Figure 2). The effect of Cu and Zn source was observed in the relative abundance of *Vibrio*, *Enterobacter*, *Propionibacterium*, and *Halomonas*, being lower for HCl than SF diets (P-adjust < 0.05; Figure 3). Meanwhile, the supplementation of Cu and Zn as HCl

Table 2. Growth performance[1] of pigs fed diets with two Cu and Zn sources (SF and HCl) at two Cu levels (15 and 160 mg/kg)[2]

Cu and Zn source	Cu level, mg/kg	BW, kg			ADFI, g			ADG, g			G: F		
		Day 0	Day 14	Day 42	Day 0 to 14	Day 14 to 42	Day 0 to 42	Day 0 to 14	Day 14 to 42	Day 0 to 42	Day 0 to 14	Day 14 to 42	Day 0 to 42
SF	15	5.86	6.97	16.62	126.6	492.6^{ab}	370.6	86.9	352.4	256.2	0.695	0.718^{b}	0.696^{b}
	160	5.86	7.37	17.99	150.5	491.4^{ab}	376.3	111.9	381.1	288.8	0.757	0.778^{a}	0.770^{a}
HCl	15	5.86	7.11	16.51	134.1	456.3^{b}	348.7	89.1	335.9	253.6	0.673	0.740^{ab}	0.729^{ab}
	160	5.86	7.36	17.45	138.5	529.0^{a}	381.0	107.0	375.6	271.2	0.780	0.714^{b}	0.718^{ab}
SEM		0.496	0.486	0.883	10.48	38.20	21.75	4.69	17.28	11.36	0.048	0.044	0.041
P-value													
Source		0.894	0.398	0.256	0.701	0.957	0.417	0.767	0.383	0.162	0.989	0.259	0.544
Level		0.894	<0.0001	0.0002	0.020	0.005	0.075	<0.0001	0.009	0.001	0.003	0.374	0.038
Source × level		0.689	0.311	0.439	0.106	0.004	0.208	0.428	0.660	0.293	0.403	0.029	0.006

[1] BW; ADFI; ADG; G:F.

[2] Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (11 pigs per replicate pen).

[a,b] Values within the same column with different letters differ significantly ($P < 0.05$).

Table 3. Antioxidant activity of pigs fed diets with two Cu and Zn sources (SF and HCl) at two Cu levels (15 and 160 mg/kg)[1]

Cu and Zn Source	Cu level, mg/kg	SOD, U/mL		GPX, U/L	
		Day 14	Day 42	Day 14	Day 42
SF		173.8	138.4	3,144	3,109
HCl		185.1	134.7	3,437	3,284
SEM		6.00	9.72	106.9	101.3
	15	179.9	143.3	3,373	3,389
	160	179.0	129.8	3,208	3,004
SEM		5.92	9.62	106.9	101.3
P-value					
Source		0.181	0.789	0.057	0.226
Level		0.921	0.328	0.277	0.013
Source × level		0.465	0.792	0.621	0.637

[1] Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (one pig per replicate pen was sampled).

Table 4. Serum and tissue Cu and Zn content of pigs fed diets with two Cu and Zn sources (SF and HCl) at two Cu levels (15 and 160 mg/kg)[1]

Cu and Zn Source	Cu level, mg/kg	Serum, mg/L[2]		Liver, mg/kg DM		Bone, mg/kg[4]
		Cu	Zn	Cu[3]	Zn	Zn
SF		1.69	0.71	1.68 (48.7)	213.6	257.2
HCl		1.79	0.72	1.80 (71.0)	221.6	268.0
SEM		0.031	0.017	0.042	9.58	3.56
	15	1.65	0.67	1.59 (39.5)	199.9	258.5
	160	1.83	0.76	1.89 (80.2)	235.4	266.7
SEM		0.031	0.017	0.041	9.53	3.72
P-value						
Source		0.037	0.438	0.036	0.553	0.041
Level		<0.0001	0.0002	<0.0001	0.011	0.115
Source × Level		0.130	0.593	0.584	0.991	0.044

[1] Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (one pig per replicate pen was sampled).
[2] P-value of day of sampling at days 14 and 42 ($P < 0.0001$). P-value of interaction between source × level × day for Cu ($P = 0.299$) and for Zn ($P = 0.010$).
[3] Log_{10}-transformed liver Cu concentration. Values in parentheses show the non-transformed values.
[4] Cu detected values are lower than 0.02 mg/g by ICP-OES.

increased the *Methanobacterium*, *Acidaminococcus*, *Gallibacterium*, *Anaerovibrio*, and *Actinobacillus* abundance compared with Cu and Zn SF (P-adjust < 0.05).

Additionally, the increase in Cu as HCl decreased the abundance of *Blautia*, *Streptococcus*, *Enterobacter*, *Fusobacterium*, *Escherichia*, and *Vibrio*, whereas SF did not (P-adjust < 0.05; Figure 4). *Lachnospira* and *Roseburia* tended to increase in pigs fed Cu at 160 mg/kg as HCl (P-adjust < 0.10; Figure 4). At nutritional level, Cu as HCl decreased the abundance of *Enterobacter*, *Pasteurella*, *Leptospira*, *Erysipelothrix*, *Vibrio*, *Actinopolyspora*, and *Clostridium*, while increasing *Lactobacillus* abundance compared with SF at the same Cu level (P-adjust < 0.05; Figure 5).

Table 5. Evenness and diversity of colon microbiota of pigs fed diets with two Cu and Zn sources (SF and HCl) at two Cu levels (15 and 160 mg/kg)[1]

Cu and Zn source	Cu level, mg/kg	Shannon	Inverse Simpson
SF	15	2.38^{ab}	4.31^{b}
	160	2.31^{b}	4.07^{bc}
HCl	15	2.54^{a}	5.18^{a}
	160	2.12^{c}	3.36^{c}
SEM		0.047	0.220
P-value			
Source		0.761	0.715
Level		<0.0001	<0.0001
Source × Level		0.0006	0.0009

[1] Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (one pig per replicate pen was sampled).
[a–c] Values within the same column with different letters differ significantly ($P < 0.05$).

Enterococcus spp. isolation, detection of antimicrobial resistance genes, and phenotypical antimicrobial resistance tests

Enterococcus species were isolated in a total of 40 samples with *E. faecalis* being the most dominating (26 samples; 65%). *E. faecium* was isolated from one sample and the remaining samples were identified as *Enterococcus* spp. (32.5%). A similar proportion

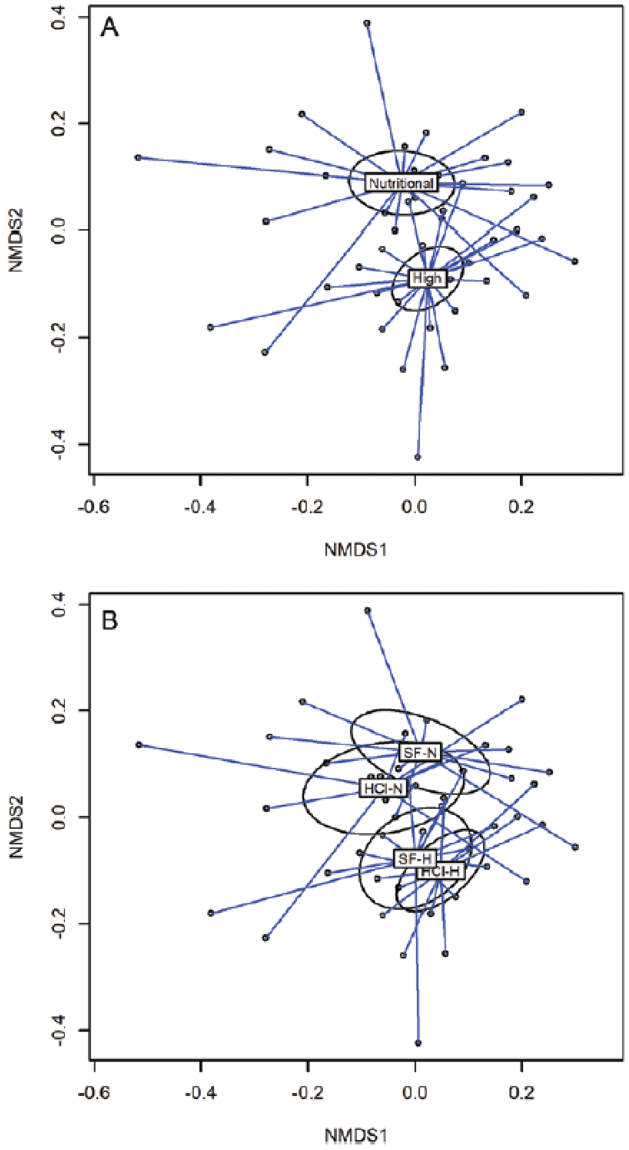


Figure 1. Nonmetric dimensional scaling (NMDS) plot of dissimilarity matrix based on Bray–Curtis distance clustered by Cu dietary level (P_{ADONIS} = 0.001) (A); and by experimental diets SF-N, sulfate at nutritional level; SF-H, sulfate at high level; HCl-N, hydroxychloride at nutritional level, HCl-H, hydroxychloride at high level (P_{ADONIS} = 0.054) (B). Data are means of 12 replicate pens for the two-way interaction, whereas for the main effect of level are means of 24 replicate pens (one pig per replicate pen was sampled).

of the different *Enterococcus* species were isolated in both HCl and SF diets (Table 6). In general, the presence of antimicrobial resistance genes in *Enterococcus* isolates was low. Only, *ermB* and *tetM* genes were detected in three and two samples, respectively (Table 6). No resistance genes were identified for Cu (*tcrB*) and VAN (*VanA*, *VanB*, *VanC1*, and *VanC2*). Meanwhile, using a disk diffusion test, all isolates were resistant to ERY, TET, CLIN, KAN, and ST and sensitive to VAN (Figure 6). The highest percentages of resistant isolates were observed for GEN (98%), CP (95%), CLOR (85%), and ENR (83%), whereas the lower resistances were observed for IMI (8%), PG (22%), and AMP (35%). The rates of antimicrobial resistance did not differ between treatments (Figure 6). Regarding MIC test results, 65% of the isolates showed MIC values between 5 and 10 mM. The mean MIC value of Cu for all isolates was 6.74 mM. No differences were observed among treatments (Table 6).

Discussion

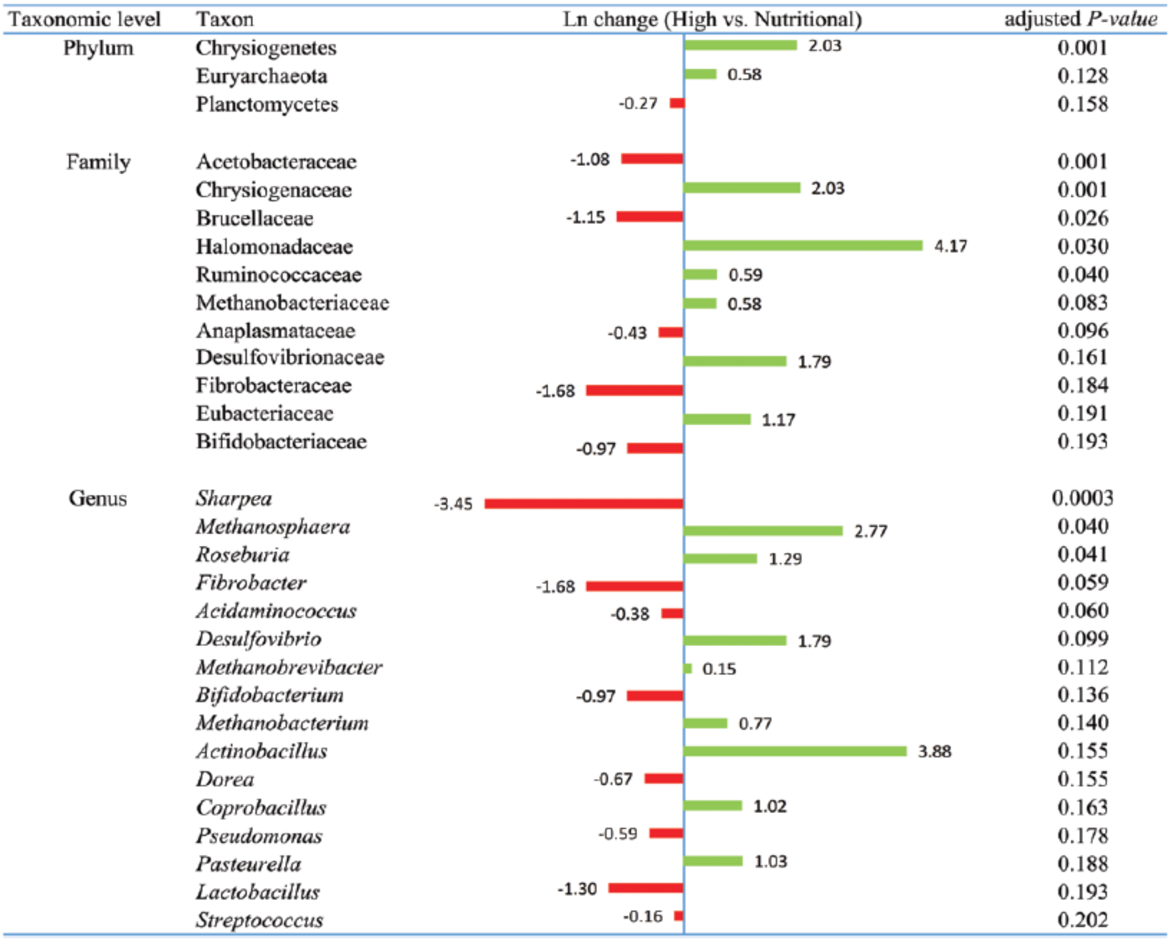
Effect of Cu level supplementation

Higher dietary Cu level increased growth performance, resulting in a difference of 1 kg BW at the end of the nursery period under challenging conditions. Suggested mechanisms for high dietary Cu level effects on performance include their effects on microbiota (Pang et al., 2009), on lipase and phospholipase activity and fat digestibility (Luo and Dove, 1996; Gonzales-Eguia et al., 2009), and on hormone production in the intermediary metabolism (Li et al., 2008) and ghrelin synthesis in the stomach (Yang et al., 2012). In fact, previous studies reported that high dietary Cu levels (160 mg/kg) in pigs' diets increased feed intake and growth performance (Bikker et al., 2015). It must be noted that in our study, weaned pigs were allotted to previously used pens that were not disinfected or cleaned in order to provide poor sanitary conditions through contact with a wide range of fecal microorganisms from older pigs. In this sense, ADG (280 g) of pigs fed the high dietary levels of Cu diets were lower than the common ADG recorded in the farm (290 g) not only in standard commercial conditions when pens were properly clean but also with the inclusion of therapeutic doses of Zn in the feed.

Different dietary levels of Cu were also associated with changes in mineral tissue concentration. High Cu levels in the diet increased the Cu and Zn content in the liver and serum. The liver is the primary storage organ and is responsible for regulating the amount of Cu and Zn in the body through bile excretion to the intestinal tract or distributing it through the blood to other organs. Therefore, the complementary evaluation of trace minerals in serum or plasma could indicate the amount of trace minerals that are circulating in the body (López-Alonso, 2012). Usually, the main antagonistic interaction between Cu and Zn has been observed when high levels of Zn in the diet (>2,000 mg/kg) are supplemented, resulting in a Cu deficiency (Gaudré, 2016). Meanwhile, dietary Cu has little or no effect on Zn metabolism (Keen et al., 1985).

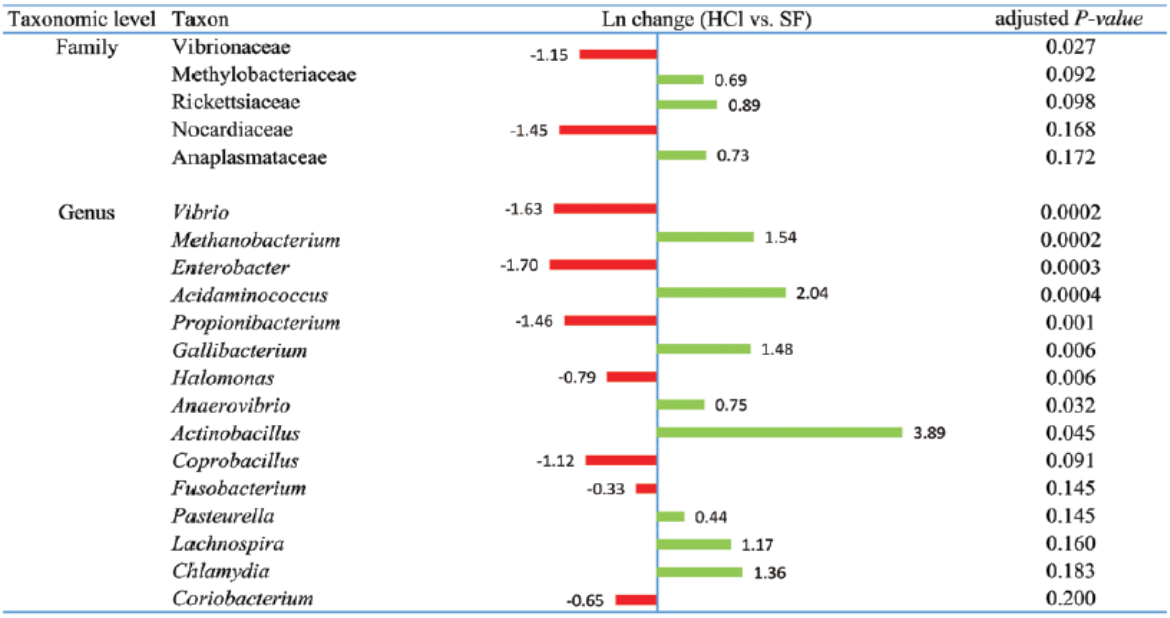
One of the primary functions of Cu is to be part of a large number of cuproenzymes in the catalysis of superoxide radicals (Suttle, 2010). Nevertheless, in excess or free unbound in the bloodstream, Cu is potentially toxic resulting in oxidation and catalyzing the formation of hydroxyl radicals (Bremner, 1998; Gaetke et al., 2014). In the present study, feeding pigs high Cu levels resulted in lower GPX activity compared with pigs fed nutritional Cu levels, suggesting that more Cu ions lead to more oxidation in plasma.

High dietary levels of Cu were also able to modify the main variable of microbiome composition. In fact, one of the growth-promoting actions of Cu has been attributed to its antimicrobial effect. Diets supplemented with Cu at 160 mg/kg decreased Brucellaceae, *Streptococcus*, and *Pseudomonas*, which may contain opportunistic pathogens, and increased Ruminococcaceae, *Actinobacillus*, and *Roseburia* compared with Cu at 15 mg/kg. The bacterial modulation, toward the reduction of opportunistic pathogens together with the development of saprophytic bacteria, could lead to a significant improvement in intestinal nutrient absorption and, therefore, pig feed efficiency. Furthermore, it is known that many members of the family and genera, which increased as a result of high Cu supplementation, produce (directly or indirectly) short-chain fatty acids (**SCFA**;



Taxonomic level	Taxon	Ln change (High vs. Nutritional)	adjusted *P-value*
Phylum	Chrysiogenetes	2.03	0.001
	Euryarchaeota	0.58	0.128
	Planctomycetes	-0.27	0.158
Family	Acetobacteraceae	-1.08	0.001
	Chrysiogenaceae	2.03	0.001
	Brucellaceae	-1.15	0.026
	Halomonadaceae	4.17	0.030
	Ruminococcaceae	0.59	0.040
	Methanobacteriaceae	0.58	0.083
	Anaplasmataceae	-0.43	0.096
	Desulfovibrionaceae	1.79	0.161
	Fibrobacteraceae	-1.68	0.184
	Eubacteriaceae	1.17	0.191
	Bifidobacteriaceae	-0.97	0.193
Genus	*Sharpea*	-3.45	0.0003
	Methanosphaera	2.77	0.040
	Roseburia	1.29	0.041
	Fibrobacter	-1.68	0.059
	Acidaminococcus	-0.38	0.060
	Desulfovibrio	1.79	0.099
	Methanobrevibacter	0.15	0.112
	Bifidobacterium	-0.97	0.136
	Methanobacterium	0.77	0.140
	Actinobacillus	3.88	0.155
	Dorea	-0.67	0.155
	Coprobacillus	1.02	0.163
	Pseudomonas	-0.59	0.178
	Pasteurella	1.03	0.188
	Lactobacillus	-1.30	0.193
	Streptococcus	-0.16	0.202

Figure 2. Differentially abundant taxa (ln change and FDR-adjusted P < 0.20) between diets supplemented at high and nutritional Cu levels, regardless of the mineral source. Positive values (green color) and negative values (red color) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa are presented. Data are means of 24 replicate pens for the main effect of level (one pig per replicate pen was sampled).



Taxonomic level	Taxon	Ln change (HCl vs. SF)	adjusted *P-value*
Family	Vibrionaceae	-1.15	0.027
	Methylobacteriaceae	0.69	0.092
	Rickettsiaceae	0.89	0.098
	Nocardiaceae	-1.45	0.168
	Anaplasmataceae	0.73	0.172
Genus	*Vibrio*	-1.63	0.0002
	Methanobacterium	1.54	0.0002
	Enterobacter	-1.70	0.0003
	Acidaminococcus	2.04	0.0004
	Propionibacterium	-1.46	0.001
	Gallibacterium	1.48	0.006
	Halomonas	-0.79	0.006
	Anaerovibrio	0.75	0.032
	Actinobacillus	3.89	0.045
	Coprobacillus	-1.12	0.091
	Fusobacterium	-0.33	0.145
	Pasteurella	0.44	0.145
	Lachnospira	1.17	0.160
	Chlamydia	1.36	0.183
	Coriobacterium	-0.65	0.200

Figure 3. Differentially abundant taxa (ln change and FDR-adjusted P < 0.20) between Zn and Cu HCl and SF diets, regardless of the Cu level. Positive values (green color) and negative values (red color) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa are presented. Data are means of 24 replicate pens for the main effect of source (one pig per replicate pen was sampled).

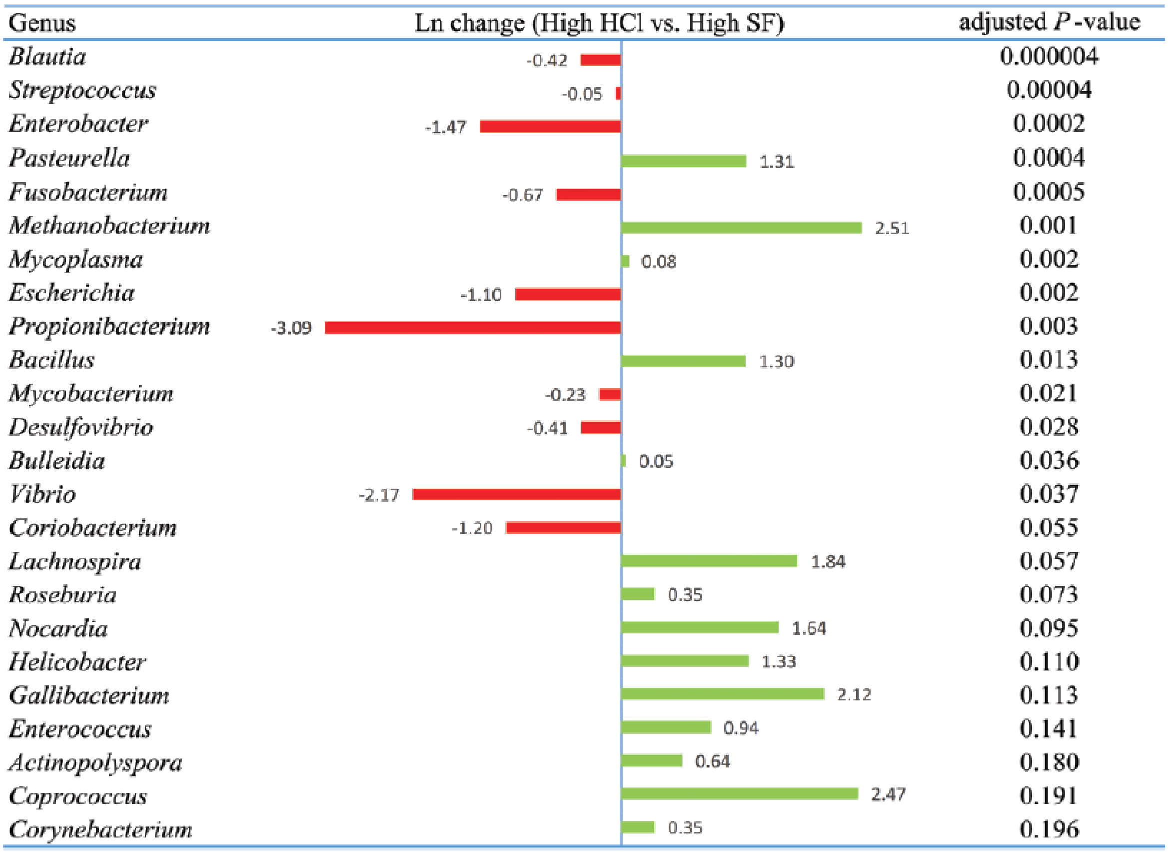


Figure 4. Differentially abundant taxa at the genus level (ln change and FDR-adjusted P < 0.20) at high Cu supplementation between HCl and SF source. Positive values (green color) and negative values (red color) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa are presented. Data are means of 12 replicate pens for the two-way interaction (one pig per replicate pen was sampled).

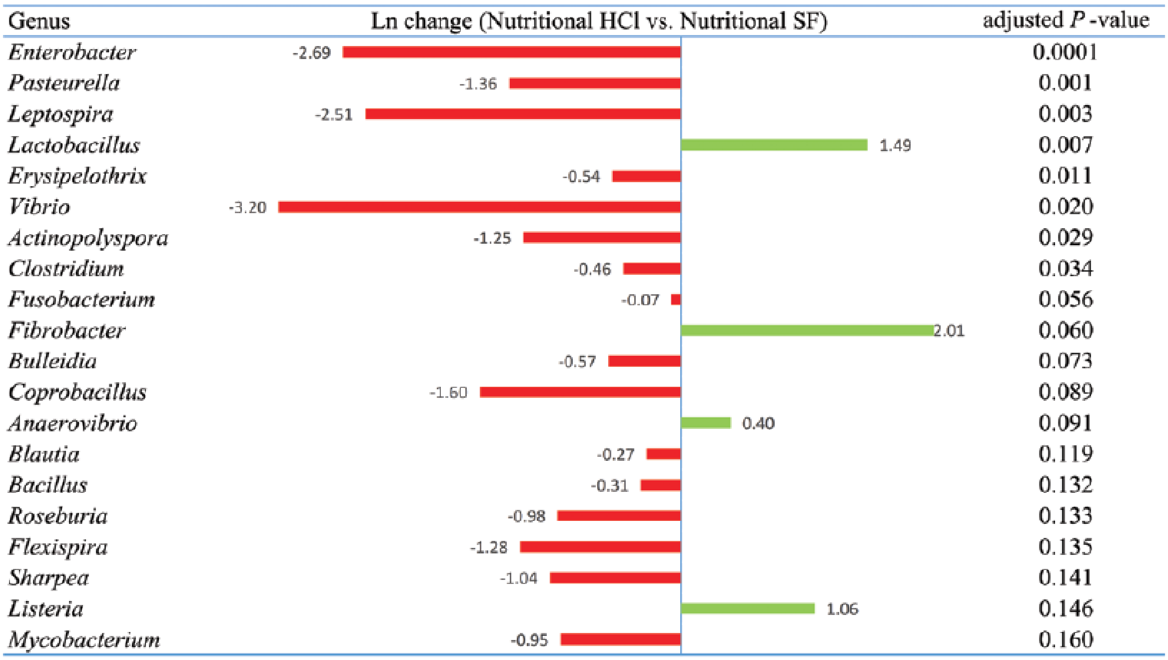


Figure 5. Differentially abundant taxa at the genus level (ln change and FDR-adjusted P < 0.20) at nutritional Cu supplementation between HCl and SF source. Positive values (green color) and negative values (red color) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa are presented. Data are means of 12 replicate pens for the two-way interaction (one pig per replicate pen was sampled).

Table 6. Characteristics of *Enterococcus* spp. isolates[1] and prevalence of antimicrobial resistance genes[2] of pigs fed diets with two Cu and Zn sources (SF and HCl) at two Cu levels (15 and 160 mg/kg)

							AMR genes[3]			
Cu and Zn source	Cu level, mg/kg	*Enterococcus* spp. isolated, *n*	*E. faecalis*, *n*	*E. faecium*, *n*	*Enterococcus* spp., *n*	Mean MIC Cu, mM	*tcrB*, *n*	*ermB*, *n*	*tetM*, *n*	*vanA*, *vanB*, *vanC1*, and *vanC2*, *n*
SF	15	11	8	1	2	5.41	0	0	0	0
	160	11	6	0	5	6.68	0	0	1 (2.5)	0
HCl	15	8	6	0	2	7.85	0	1 (2.5)	1 (2.5)	0
	160	10	7	0	3	7.05	0	2 (5)	0	0

[1] Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (one pig per replicate pen was sampled).
[2] Antimicrobial resistance genes for: Cu (*tcrB*), erythromycin (*ermB*), tetracycline (*tetM*), and vancomycin (*vanA*, *vanB*, *vanC1*, and *vanC2*).
[3] Values in parenthesis show the prevalence percentage expressed for the total *Enterococcus* spp. isolates.

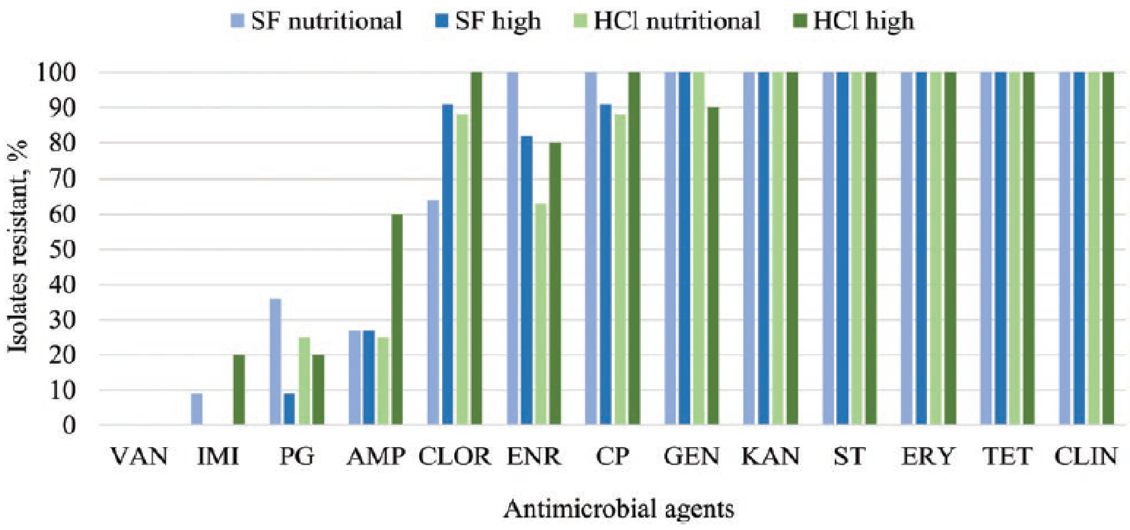


Figure 6. Percentage of *Enterococcus* spp. isolates resistant to different antimicrobials agents from pigs fed diets with two Cu and Zn sources (SF and HCl) at two Cu levels (15 and 160 mg/kg).

i.e., butyrate, propionate, and acetate) (Tungland, 2018). For instance, *Roseburia* is known to be a butyrate producer from the fermentation of dietary nondigestible carbohydrates, but in vitro studies have also shown that genera such as *Roseburia* and *Eubacterium* can use the lactate and/or acetate produced by *Bifidobacterium* to produce other SCFA as propionate (Duncan et al., 2002; Tungland, 2018). SCFA are essential forms of energy, which are rapidly absorbed by colonic epithelial cells to exert beneficial effects on the host, such as protection against colonic diseases, improvement of intestinal barrier function, and reduction of inflammation in the gut (Ríos-Covián et al., 2016). Although SCFA were not directly measured in the present study, these bacterial findings could support improved growth performance of pigs when diets are supplemented with Cu at 160 mg/kg in contrast to diets with Cu at 15 mg/kg. The association between intestinal microbiota composition of pigs and their growth performance and health has been explored in previous studies (Højberg et al., 2005; Mei et al., 2009; Yu et al., 2017).

The effect of high Cu concentration on microbial cells has been related to the induced production of intracellular reactive oxygen radicals inactivating cell components, such as nucleic acids, lipids, and proteins resulting in bacterial death (Djoko et al., 2015). However, to protect themselves from this toxic effect, bacteria evolved a range of mechanisms such as extracellular sequestration of Cu ions, relative impermeability of the outer and inner bacterial membranes to Cu ions, metallothionein Cu-scavenging proteins in the cytoplasm and periplasm, and active extrusion of Cu from the cell. The latter appears to be the chief mechanism of Cu tolerance in bacteria and has been extensively studied in Gram-positive and Gram-negative bacteria (Grass et al., 2011). Most of the Cu-scavenging proteins (CPx-type ATPases) are encoded by genes located on the chromosome. Meanwhile, Cu resistance genes are often located on plasmids, being in most cases transferable (Hasman and Aarestrup, 2002). The transferable and plasmid-located Cu resistance gene designated as *tcrB* has been identified in several *Enterococcus* species, including *E. faecium* and *E. faecalis* (Hasman and Aarestrup, 2002; Hasman et al., 2006). Interestingly, the same plasmid was also found to carry genes *ermB* and *vanA*, which encode resistance to macrolides and glycopeptides, respectively (Hasman and Aarestrup, 2002; Hasman et al., 2006). Therefore, in the present study, we focused on the detection of Cu, VAN, TET, and ERY resistance genes in *Enterococcus* spp. isolates. A total of 40 samples were identified as *Enterococcus* spp., being *E. faecalis* the most dominating (65%). From these, all isolates were negative for the *tcrB* gene. Previous studies conducted in Denmark and the United States (U.S.) reported

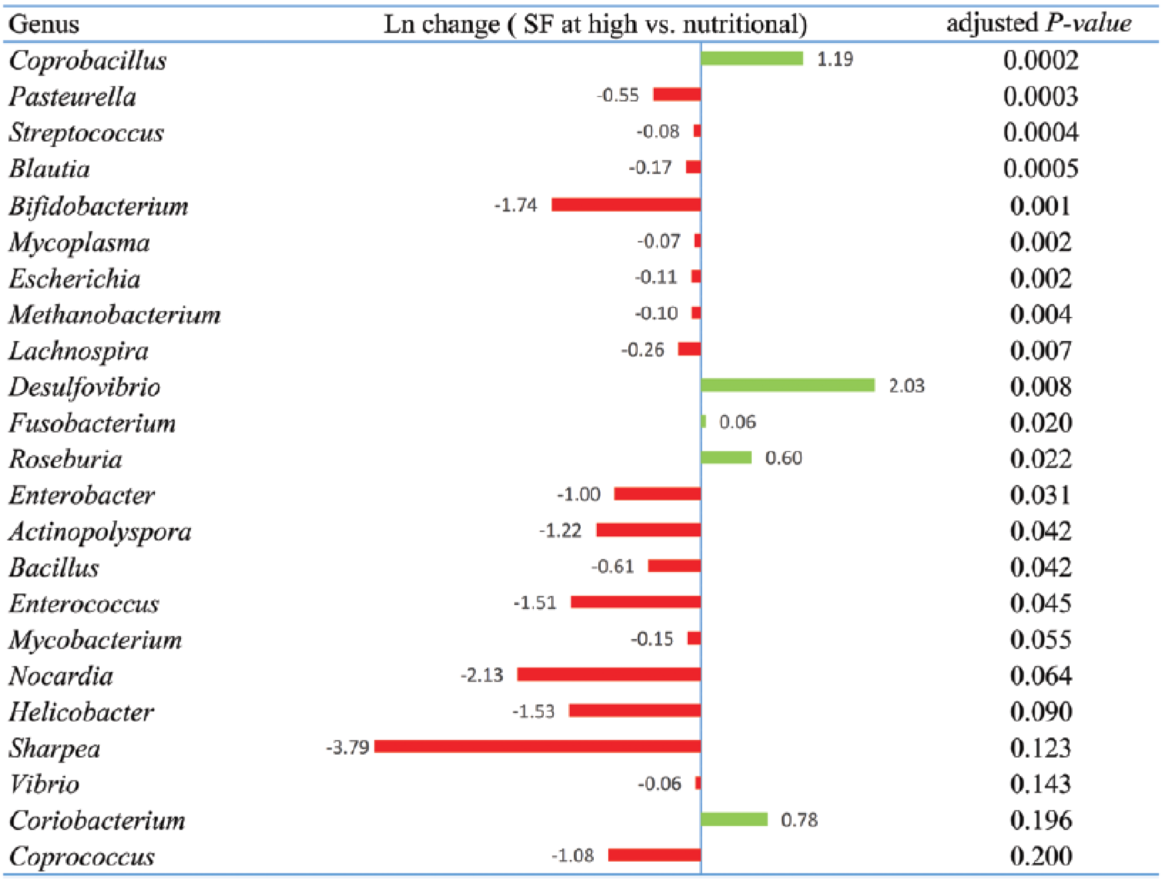


Figure 7. Differentially abundant taxa at the genus level (ln change and FDR-adjusted $P < 0.20$) between SF diets supplemented at high and nutritional Cu levels. Positive values (green color) and negative values (red color) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa are presented. Data are means of 12 replicate pens for the two-way interaction (one pig per replicate pen was sampled).

the prevalence of the *tcrB* gene in enterococcal isolates at 76% (Hasman and Aarestrup, 2002), 11.9% (Amachawadi et al., 2011) and 4.9% (Amachawadi et al., 2010) on pigs, 34% on broiler chickens (Hasman and Aarestrup, 2002), 16% on calves (Hasman and Aarestrup, 2002), and 6.9% on heifers (Amachawadi et al., 2013). It must be noted that the highest prevalence (76%) of the *tcrB* gene was described in pigs before slaughter in Denmark. The authors (Hasman and Aarestrup, 2002) point out that in Denmark, on the date when the study was performed (1998), high (165 mg/kg) concentrations of Cu SF were supplemented in weaned pigs (< 35 kg) decreasing afterward (25 mg/kg), whereas lower prevalence (Amachawadi et al., 2010, 2011) were obtained in U.S. studies feeding lower levels of Cu (16.5 and 125 mg/kg) for 35 to 42 d, similar to our study. Differences in prevalence of the *tcrB* gene reported in previous studies question whether the prevalence of the Cu resistance gene can be determined by the age of the animal, by a long-term effect of the animal's exposure (e.g., from suckling until slaughter) or by prolonged exposure of the farm to high levels of Cu. In a longitudinal study, Amachawadi et al. (2011) did not find a linear increase (days 0, 14, 28, and 42) in the prevalence of *tcrB*-positive fecal enterococci in weaned pigs fed diets with a continued supplementation of low (16 mg/kg) or high (125 mg/kg) level of Cu for 42 d. Further longitudinal field studies are required to elucidate the effect of high levels of Cu in the diet in the presence of the *tcrB* gene in animals in a farm environment. The absence of the *tcrB* gene in our isolates agrees with the low MIC Cu results (<10 mM). From the literature, it can be drawn that *tcrB*-positive *Enterococcus* are associated with MIC > 20 mM/Cu, while those *tcrB*-negative isolates had values <8 mM/Cu (Hasman and Aarestrup, 2002; Amachawadi et al., 2010, 2011, 2013). No resistance genes for *vanA*, *vanB*, *vanC1*, and *vanC2* were detected, and all isolates were phenotypically susceptible to VAN. Surprisingly, the prevalence of *ermB* (7.5%) and *tetM* (5%) genes was low as opposed to the phenotypically resistant results to ERY and TET. In *Enterococcus* spp., the most common genes conferring resistance to antibiotics are for ERY, TET, and VAN (Oravcova et al., 2019; Tian et al., 2019). Nevertheless, it is possible that this phenotypical resistance is conferred by other mechanisms or untested genes. The high rates in phenotypical resistance of enterococcal isolates could be explained by the fact that the north-east of Spain has one of the densest pig populations in Europe and different antimicrobial agents are still widely used in livestock. Moreover, a long-term effect on the microbial population after antibiotic administration should be considered. In pigs, the effects of a single intramuscular administration of amoxicillin may persist at least after 5 wk (Janczyk et al., 2007). Although the potential selective pressure that Cu supplementation could exert on antimicrobial resistance was not evidenced in the present study, it certainly requires more attention.

Effect of Cu and Zn source supplementation

In the present study, differences between mineral sources were observed in growth performance (ADFI and G:F), mineral tissue content, and microbial community. Results of studies with broiler chickens indicate that the effect of Cu and Zn on growth

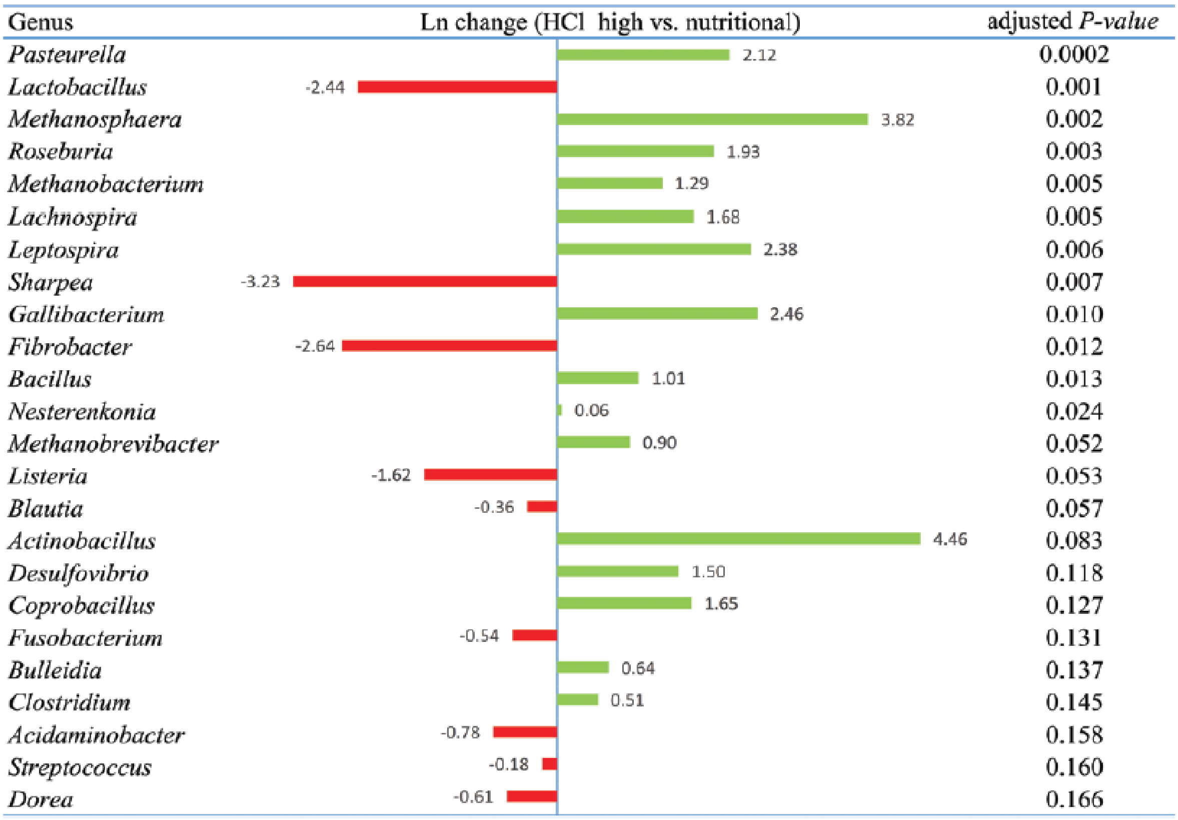


Figure 8. Differentially abundant taxa at the genus level (ln change and FDR-adjusted $P < 0.20$) between HCl diets supplemented at high and nutritional Cu levels. Positive values (green color) and negative values (red color) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa are presented. Data are means of 12 replicate pens for the two-way interaction (one pig per replicate pen was sampled).

performance could depend on chemical differences between trace mineral sources. Olukosi et al. (2018) reported that broiler chickens receiving Zn and Cu HCl had greater G:F than those fed Zn and Cu SF. Likewise, Lu et al. (2010) described that broiler chickens fed 200 mg/kg Cu as Tribasic copper chloride (**TBCC**) had greater ADG than those fed Cu SF. Similarly, supplementation of diets for broiler chickens with Cu SF at 300 mg/kg had reduced ADG and reduced G:F ratio than birds fed Cu_2O at the same level (Hamdi et al., 2018). Results of pig studies have demonstrated that other forms of both Cu and Zn, such as lysine complex (Coffey et al., 1994; Apgar et al., 1995; Cheng et al., 1998) and HCl (Cromwell et al., 1998; Fry et al., 2012; Carpenter et al., 2016), are as effective in improving growth as SF minerals. Although different studies on pigs have shown no differences between Cu and Zn sources at high or low levels, the evaluation of intermediate levels might reveal differences that are mainly driven by the higher or lower bioavailability of trace mineral sources. In this sense, Veum et al. (2004) reported that feeding pigs with intermediate Cu levels (25, 50, and 100 mg/kg) as Cu proteinate had higher ADG and ADFI than those fed a high level of Cu SF (250 mg/kg). The possibility of reducing the amounts of Cu and Zn by using higher bioavailable sources could represent an alternative to the inclusion of pharmaceutical doses of trace minerals in diets. Therefore, the negative interactions between nutrients and the environmental impact attributed to high doses could be reduced without affecting pig performance. In the context of stricter regulations, further studies exploring the bioavailability through increasing doses of different mineral sources should be explored, particularly to suckling and weaned pigs.

Pigs fed with HCl minerals had higher Cu concentrations in liver and serum compared with SF. An important factor for intestinal absorption of Cu and Zn is their availability as free ions in the intestinal lumen (Martin et al., 2013). Results from our laboratory confirmed that SFs are highly soluble in a wide range of pH from 2.5 to 6.5, whereas HCl minerals are less soluble at pH 6.5 but highly soluble at pH 2.5, as previously reported (Pang and Applegate, 2006). Consequently, less chelated interactions with other components of the diet may have occurred with HCl minerals, making them more available to be absorbed compared with SF. The fact that pigs fed a high level of Cu as SF had lower Zn content in the tibia could suggest a likely antagonistic interaction between high Cu level and Zn, and possibly with other minerals, for metal-binding sites that HCl minerals did not show. Results from earlier studies reported differences and interactions between Cu and Zn sources in absorption and mineral tissue accumulation. For instance, in broiler chickens, Olukosi et al. (2018) reported that Cu liver was influenced by the Cu and Zn source being greater for HCl than for SF minerals. Huang et al. (2015) reported greater Zn and Cu storage in the liver of pigs fed Cu as TBCC compared with those fed Cu as SF. Further studies involving complementary analysis of both protein and mRNA levels of Cu and Zn transporters could help to clarify differences in mineral storage as well as lead to a more comprehensive understanding of metal absorption pathways.

After 14 d of weaning, a tendency for higher serum GPX activity in pigs fed Cu and Zn HCl was observed. Highly soluble trace mineral sources may result in greater oxidation rates (Miles et al., 1998). Earlier studies in broiler chickens reported that TBCC was less active than SF in promoting oxidation of vitamin E in feed and in reducing vitamin E content in plasma and liver (Luo et al., 2005; Lu et al., 2010). Results of studies with pigs demonstrated that Cu SF diet at 225 mg Cu/kg may cause greater oxidative stress in the duodenum than Cu as TBCC (Fry et al., 2012; Huang et al., 2015). The covalent bonding of HCl trace minerals could allow Cu and Zn to gradually become soluble in the small intestine, thus resulting in less oxidative stress than the SF counterparts, as suggested by Fry et al. (2012).

Since mineral sources have different solubility, they may affect the intestinal microbiota differently. In our study, an increase in the relative abundance of some beneficial bacteria was observed in pigs fed HCl minerals, particularly at high Cu level. From the literature, it is known that gut microbiota plays essential roles in amino acid catabolism and energy harvest from the diet. Indeed, genera such as *Lachnospira*, *Roseburia*, and *Coprococcus* produce various metabolites such as SCFA and biogenic amines (Tungland, 2018). Based on these results, HCl diets appear to improve the intestinal microbiota profile and some mineral content in tissues. High levels of Cu as HCl increased BW performance; however, the beneficial effects of HCl were not completely reflected in pig feed efficiency compared with high Cu SF. Additional markers such as fecal consistency score and intestinal integrity indicators, which were not measured in the present study, are needed to draw consistent conclusions.

No differences in the presence of antimicrobial resistance genes or phenotypical antimicrobial resistance profile between Cu and Zn sources were observed. Nevertheless, this relationship should be discussed in field studies in greater depth and with a greater number of *Enterococcus* spp. isolated.

In conclusion, the EU permissible levels of Cu (160 mg/kg) increased growth performance and modulated bacterial communities compared with nutritional levels (15 mg/kg) in weaned pigs reared under challenging conditions. Different effects on mineral tissue content and microbial modulation were observed between Cu and Zn sources. The reduction of Cu and Zn contents in pig diets by using higher bioavailable sources should be explored in order to reduce the environmental impact. Longitudinal field studies are necessary to confirm the influence of high levels of Cu supplement on antimicrobial cross-resistance genes.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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CHAPTER IV

Dietary Preference of Newly Weaned Pigs and Nutrient Interactions According to Copper Levels and Sources with Different Solubility Characteristics

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Article

Dietary Preference of Newly Weaned Pigs and Nutrient Interactions According to Copper Levels and Sources with Different Solubility Characteristics

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Simple Summary: Strategies for promoting early feed acceptance and avoiding pig rejection to new feed are a priority for maximizing their feed intake. Animal preference or aversion for a particular feed or nutrient is a behavioral expression coordinated by a complex biological system. After weaning, Cu blood level decrease, which is probably intensified by a low feed intake. This can lead to suboptimal Cu level for the normal functioning of the body. In the present study two experiments were performed to assess the pig Cu preference. In Exp.1 (dose preference) pigs were given a choice between diets supplemented with Cu at 15 mg/kg or 150 mg/kg. In Exp.2 (source preference) diets supplemented with Cu at 150 mg/kg were offered with either sulfate or hydroxychloride source. An in vitro assay was performed to determine the Cu solubility of each source in similar conditions to those found in the oral cavity and digesta. Our results show that pigs chose diets with higher Cu levels, probably to re-establish homeostasis after weaning. Pigs preferred diets with Cu hydroxychloride compared to Cu sulfate, probably due to their solubility differences. A better understanding of pig feed preferences after weaning and their feeding behavior would improve early feed acceptance.

Abstract: Two feeding preference experiments and an in vitro assay were performed to assess the weaned pig preference for Cu doses and sources based on their sensorial perception and on the likely post-ingestive effects of Cu. At day 7 post-weaning, a total of 828 pigs were distributed into two different experiments. In Exp.1 (dose preference) a diet with a nutritional Cu level (15 mg/kg) of Cu sulfate (SF) was pair offered with higher Cu levels (150 mg/kg) of either SF or hydroxychloride (HCl). In Exp.2 (source preference), a diet supplemented with Cu-SF at 150 mg/kg was compared to a Cu-HCl (150 mg/kg) diet. At the short-term (day 7–9) and for the entire experimental week (day 7–14), pigs preferred diets with a high Cu level than with Cu at a nutritional dose ($p < 0.05$). Likewise, pigs preferred diets supplemented with a Cu-HCl source compared to diets with Cu-SF ($p < 0.05$). In vitro assay results showed a greater solubility and interaction of Cu-SF with phytic acid compared to Cu-HCl. In conclusion, pigs chose diets with higher levels of Cu probably to re-establish homeostasis after weaning. Pigs preferred diets with Cu-HCl compared to Cu-SF probably due to their solubilities and chemical differences.

Keywords: copper; feed preference; levels; post-ingestive effect; sources; solubility

1. Introduction

Weaning is considered the most critical period in swine production due to low feed intake, gastrointestinal disturbances, impaired gut integrity and consequently growth. Strategies for promoting early feed acceptance and avoiding feed neophobia and rejection after weaning are a priority for maximizing feed intake.

Animal preference or aversion for a particular feed or nutrient is a behavioral expression orchestrated by a complex biological system that involves different organs and sensory, metabolic and physiological feedbacks [1,2]. Several studies have confirmed that pigs have the ability to select among dietary ingredients and/or nutrients, for example, types of cereals [3] and amino acids [4]. Pigs have also been found to modulate their feed preference under nutrient deficit scenarios in order to re-establish homeostasis [5,6]. In terms of mineral nutrition, the swine industry usually includes high quantities of trace minerals such as Cu or Zn in pig diets due to their beneficial effects for controlling gastrointestinal dysbiosis and increasing feed intake [7]. To our knowledge, only one previous study has assessed the preference of finishing pigs (body weight; BW: 86 kg, approximately) for diets without Cu supplementation or diets with Cu at 150 mg/kg [8]. After weaning, blood levels of some trace minerals, such as Cu (1.8 mg/L vs. 1.4 mg/L) and Zn (1.10 mg/L vs. 0.76 mg/L), may decrease [9,10]. This, together with the low feed intake after weaning, may lead to suboptimal Cu and Zn levels for a proper metabolic and immune response. Since the preference for a diet may also be influenced by the animals' nutritional requirements [6,11], it is relevant to gain a better understanding of weaned pigs' preferences and acceptance for trace minerals within the range of nutritional and high inclusion levels, as well as different commercial sources.

Studies with humans have concluded that the taste of Cu in water depends on its chemical structures and its solubility [12,13]. Since pigs have approximately three times more taste buds than humans [14], it is likely that pigs have superior taste perception. Cu sulfate pentahydrate is a widely used source in pig diets. It is characterized by a high solubility in water and acid solutions and hence higher interactions with other components of the diet, including phytic acid and other minerals [15–17]. Phytic acid derived from plant ingredients can easily bind divalent metal ions such as Cu, resulting in mineral–phytin complexes and reducing the solubility of Cu and P [16]. Another common Cu source is Cu hydroxychloride. This has a crystalline structure formed by covalent bonds that gives it low solubility above pH 4 and insolubility in water [16].

The present study was carried out to test the hypothesis that weaned pigs will be able to select feed based on the sensorial perception of Cu sources due to differences in their solubility, and that pigs will choose feed based on the likely post-ingestive effects of Cu, resulting in a preference or aversion for feed. We also hypothesized that the effective solubility parameters will also depend on the interaction between Cu and phytic acid, as these are different among Cu sources. Thus, the aim of the study was to evaluate the preference of weaned pigs for diets that contain added Cu at 15 mg/kg (nutritional) and 150 mg/kg (high) as inclusion levels (dose preference experiment, Experiment (1) from the source of either Cu sulfate or Cu hydroxychloride (source preference experiment, Experiment (2). In addition, an in vitro assay was performed to determine trace mineral properties in similar conditions to those found in the oral cavity and digestive tract by testing in vitro the Cu solubility of each source (at 50, 100, 200 and 300 mg Cu/L) and its interaction with phytic acid, in buffer solutions at pH 2.5, 4.5 and 6.5. Together, the animal feed experiments, and the in vitro assay are intended to describe some clues of pig preference for different Cu levels (15 vs. 150 mg/kg) and sources (sulfate vs. hydroxychloride). In the establishment of the methodology of the present study some points were considered, such as a suitably methodology for evaluate animal preference, measurement points, length of phases and weaning age of animals under commercial conditions, as well as the simultaneous comparison between nutritional and high Cu doses in newly weaned pigs. All these considerations make the present study different from previous related works on mineral nutrition.

2. Materials and Methods

2.1. Ethics Statement

All animal experimentation procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona in compliance with the European Union guidelines for the care and use of animals in research (approval code CEEAH2788M2) [18].

2.2. Feed Preference Experiment (Experiment 1 and 2)

2.2.1. Animals and Housing

The present preference experiments were performed under Spanish commercial conditions. A total of 828 entire male and female pigs ((Large White × Landrace) × Pietrain) were selected to be used in two different preference experiments. Pigs were weaned at day 21 of age and housed in a weanling room belonging to the same commercial farm. At day 7 after weaning, pigs were blocked by sex and distributed, according to a homogenous BW, into different experimental treatments. In the first double choice test (dose preference), 552 pigs (initial BW: 6.6 ± 1.10 kg) were allocated to 24 pens (23 pigs/pen) randomly assigned to two different treatments (12 replicate pens/treatment). In the second double choice test (source preference), 276 pigs (initial BW: 7.1 ± 1.08 kg) were allocated to 12 pens (23 pigs/pen/treatment). Pens of males and females were assigned equally to dietary treatments. Each pen (4.04 m^2) was equipped with two commercial pan feeders (Maxi hopper, Rotecna, Spain) and a nipple drinker to provide ad libitum access to feed and water. The facility was environmentally controlled (temperature and ventilation rate) using thermostatically controlled heaters and exhaust fans.

2.2.2. Experimental Design and Dietary Treatments

In order to avoid biases due to the low feed consumption of newly weaned pigs during the first week after weaning (day 1–7 post-weaning), pigs were adapted to the new environmental commercial conditions by offering ad libitum a commercial pre-starter diet including 9 mg Cu/kg. The preference test was performed during the second week (day 7–14) after weaning. At day 7 post-weaning, pan hopper feeders used during the adaptation phase were completely emptied and replaced by two new pan hopper feeders to provide the reference diet and the assigned experimental diet in each pen. In order to avoid biases, both pan hopper feeders were hand filled to ensure completely free access to the two diets. The position of the feeders (right or left) inside the pen was switched once (day 9) to prevent location bias in feeding behavior.

In the dose preference test, a diet with a nutritional Cu level (15 mg Cu/kg) of Cu sulfate pentahydrate (Pintaluba, Reus, Spain) was used as a reference diet and was pair offered with higher Cu levels (150 mg/kg) of either Cu sulfate or Cu hydroxychloride (IntelliBond C, Trouw Nutrition, the Netherlands). In contrast, in the source preference test, the reference diet was supplemented with Cu (150 mg/kg) as sulfate pentahydrate and compared to a Cu hydroxychloride (150 mg/kg) diet. Supplementation of Zn (Sulfate monohydrate; Pintaluba, Reus, Spain) was fixed for all diets at 100 mg/kg, according to National Research Council (NRC) [19] requirements. Feed was offered in pellet form and both feed and water were provided ad libitum. A vitamin–mineral premix without Cu was prepared. For each dietary treatment, Cu products were pre-mixed with 25 kg of basal diet before being put directly in the mixer during the feed preparation process. The composition of the basal diet is shown in Table 1. Diets contained 500 units of phytase (Axtra PHY TPT, Danisco, Marlborough, UK) per kg of complete feed.

Composite samples (1 kg) were collected during the bagging process as representations of each experimental treatment. Each sample was therefore split proportionally into four 250 g samples that were stored for further analysis. Zinc oxide was not added at pharmacological levels to the diets and no antibiotics or feed additives with flavoring or antimicrobial properties were used.

Table 1. Composition of the basal diet for Cu preference experiments, as-fed basis.

Ingredients, %	Basal Diet
Wheat	30.00
Maize	23.23
Soybean meal 47% Crude protein	13.85
Sweet milk whey	11.07
Barley	9.00
Soybean meal heat treated	4.00
Fishmeal	2.50
Lard	1.80
Porcine plasma	1.50
Calcium carbonate	0.73
Mono-calcium phosphate	0.25
L-Lysine 50	0.74
DL-Methionine	0.22
L-Threonine	0.21
L-Valine	0.13
L-Tryptophan	0.07
Salt	0.30
Vitamin mineral premix [1]	0.40
Calculated composition, %	
Dry matter	89.08
Net energy, kcal/kg	2460
Crude protein	19.22
Neutral detergent fiber	8.47
Ether extract	4.72
Ca	0.60
Total P	0.52
Dig P	0.30
Analysed composition, % [2]	
Dry matter	91.15
Crude protein	19.55
Neutral detergent fiber	9.68
Ether Extract	4.04
Ash	4.62

[1] Provided per kg of feed: vitamin A (acetate): 12,000 IU; vitamin D3 (cholecalciferol): 2000 IU; vitamin E: 75 IU; vitamin K3: 2 mg; vitamin B1: 3 mg; vitamin B2: 7 mg; vitamin B6: 7.3 mg; vitamin B12: 0.06 mg; D-pantothenic acid: 17 mg; niacin: 45 mg; biotin: 0.2 mg; folacin: 1.5 mg; Fe (chelate of amino acid): 80 mg; Zn (sulphate pentahydrate): 100 mg; Mn (dimanganese chloride trihydroxide): 45 mg; I (calcium anhydrous): 0.7 mg; Se (sodium): 0.3 mg; butylated hydroxytoluene (BHT): 2 mg. Phytase: 500 FTU (Axtra PHY TPT, Danisco, Marlborough, UK). [2] Analyzed Cu content in experimental diets: Cu sulfate pentahydrate (29 mg/kg), Cu sulfate pentahydrate (154 mg/kg) and Cu hydroxychloride (152 mg/kg).

2.2.3. Experimental Procedures and Measurements

Feed intake for the reference and the experimental diets was measured from day 7–9 (short-term preference), and from day 9–14 (long-term preference). The methodology and reference periods were defined from the works of Forbes [6], Torrallardona and Solà-Oriol [20] and Roura et al. [11]. Thus, the short-term preference (2 d in the present study) is considered as the immediate response of animals to the different sensory characteristics of the feed, whereas the long-term preference (5 day in the present study) might be defined as more than a few days determined by learned preferences and aversions according the metabolic response of animals to the feed. The feed intake values of each diet were expressed as described in [21] but with a modification. Briefly, feed intake per replicate pen was standardized by dividing the diet feed intake by the average pig body weight and by the number of pigs per replicate pen. Then, the preference of the test diet relative to the reference diet was calculated as the percentage contribution of the test diet to the total feed intake as described in [3]. Preference values can range between 100 and 0%. A value of 50% would indicate indifference with respect to the reference diet, whereas values significantly higher or lower than 50% would indicate a significant preference or aversion, respectively.

2.3. *In Vitro Assay*

The solubility of Cu from a sulfate or hydroxychloride source and the concentration of soluble phytic phosphorus (PP) were measured in duplicate. Cu was added at concentrations of 50 mg/L, 100 mg/L, 200 mg/L and 300 mg/L in 200 mM glycine buffer (pH 2.5) (Merck, Germany) and 200 mM sodium acetate buffer (pH 4.5 and 6.5) (Merck, Germany). Each Cu source was mixed with 20 mL of buffer with and without 2.9 mM phytic acid (Merck, Germany), incubated at 41 °C in a shaking water bath for 1 h and filtered through 42 µm Whatman filter paper. Soluble Cu and PP analysis was carried out with inductively coupled plasma-optical emission spectroscopy (ICP-OES, model Optima 4300 DV, PerkinElmer Inc.; Waltham, MA, US). The solubility of PP was expressed as mg/L, whereas the solubility of Cu was calculated using the following equation (1):

$$\text{Mineral Solubility } (\%) = (\text{Soluble mineral})/(\text{Total mineral}) \times 100 \tag{1}$$

2.4. *Statistical Analysis*

Standardized preference percentage values were analyzed as a randomized complete block design using the MIXED procedure of SAS (version 9.4, SAS Institute; Cary, US). The model included the fixed effects of treatment and the random effects of sex. The pen was considered the experimental unit. The normality and homogeneity of the data were examined with the Shapiro–Wilk statistical test of SAS® before statistical analysis. In addition, the preference percentage values of each experimental diet were compared to the neutral value of 50% using a Student's T-test procedure. Significantly different means were separated using the Tukey adjust. Significance was defined at a probability $p \leq 0.05$ and tendencies were considered when *p*-values were between >0.05 and <0.10.

3. Results

The analyzed mineral concentrations in feed are shown in Table 1. The expected difference between nutritional and high levels of Cu in the diets was achieved with both sulfate and hydroxychloride Cu sources.

The growth performance of pigs for preference experiments are shown in Table 2. Since the two experimental diets were provided at the same time in each pen, differences in growth performance cannot be attributed to either diet.

Table 2. Growth performance of pigs in Cu preference experiments [1].

Item	Growth Performance [2]		
	BW (7 Day), kg	BW (14 Day), kg	ADG, g
Cu dose preference [3]			
Comparison (a)	6.63 (±1.286)	7.76 (±1.255)	160.9 (±34.80)
Comparison (b)	6.66 (±0.931)	7.81 (±1.034)	165.1 (±63.36)
Cu source preference [4]	7.08 (±1.078)	8.38 (±1.155)	184.4 (±32.26)

[1] Data are means of 12 pens with 23 pigs per replicate pen. [2] Body weight, BW; Average daily gain, ADG. Values in parenthesis indicate the standard deviation of means.[3] Dose preference test between Cu sulfate at 15 mg/kg and Cu sulfate at 150 mg/kg (a) and between Cu sulfate at 15 mg/kg and Cu hydroxychloride at 150 mg/kg (b). [4] Source preference test of Cu at 150 mg/kg of either Cu sulfate or Cu hydroxychloride.

3.1. *Dose Preference Test (Exp. 1)*

The pig preference response for the experimental diets is shown in Table 3. In the short-term, when pigs were given a choice between diets supplemented with nutritional or high Cu level, they preferred high Cu diets, regardless of the source ($p < 0.05$). In the long-term period, the feed preference was not different between experimental diets ($p < 0.10$). Considering the entire experimental period, pigs preferred diets supplemented with high Cu level with a hydroxychloride source than those supplemented with a nutritional Cu level (58.9% vs. 41.1%; $p = 0.035$).

Table 3. Feed preference percentage at short-term period (day 7–9), long term period (day 9–14) and for the entire experimental period (day 7–14) for Cu preference experiments [1].

Item	Cu Level, mg/kg	Preference Percentage		
		Short-Term	Long-Term	Entire Trial
Cu dose preference [2]				
Comparison (a)				
Sulfate	15	36.04 *	44.16	41.80 *
Sulfate	150	63.96 *	55.84	58.20
SEM [4]		4.692	7.985	6.030
p-value		0.0004	0.312	0.068
Comparison (b)				
Sulfate	15	23.49 *	47.69	41.12 *
Hydroxychloride	150	76.51 *	52.31	58.88
SEM		5.646	8.351	5.589
p-value		0.0001	0.698	0.035
Cu source preference [3]				
Sulfate	150	37.76 *	43.71	42.71
Hydroxychloride	150	62.24 *	56.29	57.29
SEM		5.456	5.838	4.693
p-value		0.004	0.141	0.039

[1] Data are means of 12 pens with 23 pigs per replicate pen. [2] Dose preference test between Cu sulfate at 15 mg/kg and Cu sulfate at 150 mg/kg (a) and between Cu sulfate at 15 mg/kg and Cu hydroxychloride at 150 mg/kg (b). [3] Source preference test of Cu at 150 mg/kg of either Cu sulfate or Cu hydroxychloride. [4] Standard error of the mean, SEM. * Asterisks indicate that experimental diet is significantly different ($p < 0.05$) to the preference neutral value (50%) using T–test.

3.2. Source Preference Test (Exp. 2)

Pig preference for Cu sources is shown in Table 3. In the short-term, pigs showed a greater preference for diets supplemented with the Cu hydroxychloride source than those with the Cu sulfate (62.2% vs. 37.8%; $p = 0.004$). In the long-term period, no differences were observed ($p = 0.141$). Considering the entire experimental period, pigs preferred diets supplemented with Cu hydroxychloride compared to those supplemented with Cu sulfate (57.3% vs. 42.7%; $p = 0.039$).

3.3. In Vitro Assay

The results of Cu solubility and interaction with phytic acid of the two Cu sources are shown in Figures 1–3. As shown in Figure 1, the mean Cu solubility of Cu sulfate, as an average of the four concentrations, was 98% in the whole range of pH levels (2.5–6.5), whereas the hydroxychloride Cu solubility, as an average of the four concentrations, decreased drastically from 100% at pH 2.5 and 4.5 to 9% at pH 6.5.

Overall, the presence of phytic acid decreased the Cu solubility as pH increased (from 99% at pH 2.5 to 21% at pH 6.5; Figures 2 and 3), regardless of the source. At pH 2.5, the solubility of Cu was similar for both sources and it was not modified by the presence of phytic acid in samples (Figure 2).

At pH 4.5 the addition of phytic acid decreased the mean Cu solubility of Cu sulfate from 100% (without phytic acid) to 88% (Figure 3a). Likewise, the mean Cu solubility of Cu hydroxychloride decreased from 100% to 56% when phytic acid was added (Figure 3a). At intestinal pH (6.5), the presence of phytic acid did not affect Cu solubility of Cu hydroxychloride but sharply reduced Cu solubility of Cu sulfate (Figure 3b).

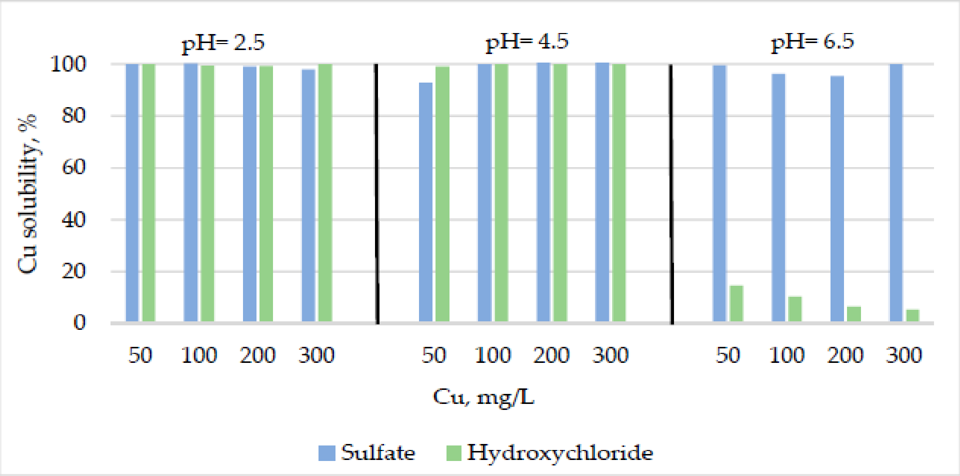


Figure 1. Effects of Cu source and level on Cu solubility at pH 2.5, 4.5, and 6.5 in the absence of phytic acid.

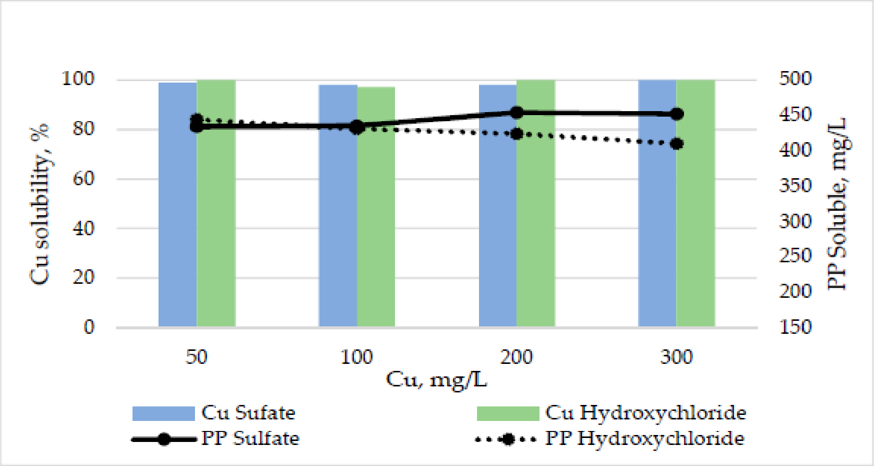
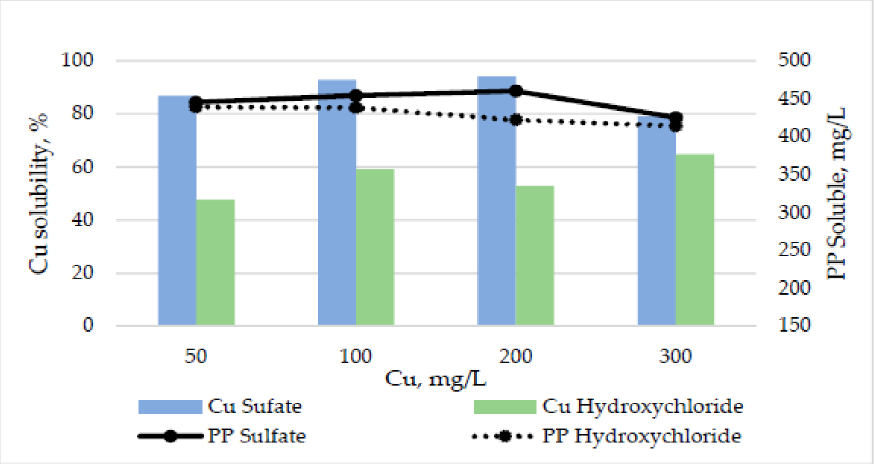
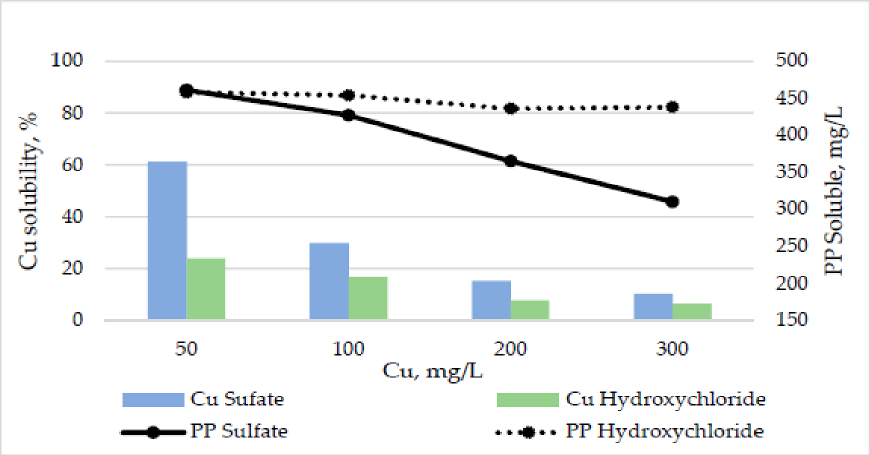


Figure 2. Effects of Cu source and level on Cu solubility and soluble phytic phosphorus (PP) content at pH 2.5 in the presence of phytic acid.



(**a**)

Figure 3. *Cont.*



(b)

Figure 3. Effects of Cu source and level on Cu solubility and soluble phytic phosphorus (PP) content at pH 4.5 (a) and 6.5 (b) in the presence of phytic acid.

In general, soluble PP decreased as the pH and the Cu dose increased (Figures 2 and 3). At pH 2.5 and 4.5 no substantial differences between the two Cu sources were observed in soluble PP content (Figures 2 and 3a), whereas at pH 6.5, increasing levels of Cu with a sulfate source, but not with a hydroxychloride source, notably reduced the content of soluble PP (Figure 3b).

4. Discussion

The average daily feed intake (ADFI) of pigs during the experimental week was within the commercial standard values, and were 201 g and 204 g for the dose and source preference studies, respectively. One of the proposed hypotheses of the present study was that weaned pigs might be able to select feed based on the likely post-ingestive effects of Cu on pigs. Our results showed that when pigs are given a choice, they preferred diets supplemented with higher Cu levels over those with nutritional levels.

In pig life, weaning is considered one of the most difficult phases. Two phases can be distinguished after weaning based on the changes in feed intake and the subsequent impacts on the physiology of the gastrointestinal tract: an acute phase (within the first 5–7 days after weaning) and an adaptive phase (day 7 after weaning and onwards) [22]. The temporary low feed intake (below 100 g/day) during the acute phase may create a deficiency in macronutrients, micronutrients and a negative energy balance, which can impair health, development and recovery during the adaptive phase [23,24]. For instance, Carlson et al. [7] reported that Cu plasma status decreased during the first two weeks after weaning, from 1.8 mg/L to 1.4 mg/L. The NRC [19] established a minimum requirement of Cu (6 mg) per kg of diet or as a daily Cu requirement (1.6–2.8 mg) according to the BW (5–11 kg) of pigs. During the lactation period, it is assumed that the consumption of milk meets the suckling pigs' daily Cu physiological requirement. However, to meet this Cu requirement after weaning, pigs must consume 267 g feed/day if feed contains 6 mg/kg as recommended by the NRC. As mentioned before, after pigs are weaned, they have an extremely low feed intake, which may promote this post-weaning Cu deficiency. In the present study during the first week after weaning, pigs were offered a commercial diet with Cu supplementation (9 mg/kg) above NRC recommendations [19], but it was probably insufficient during the critical first period after weaning. Although the serum Cu status of pigs after weaning was not measured, our results suggest that pigs' preference for diets supplemented with high levels of Cu is probably a systemic response to restore Cu levels after weaning. In this sense, Bikker et al. [7] reported that after 56 days of supplementation, pigs fed low Cu diets (15 mg/kg) had higher mRNA levels of Cu transporters in their upper small intestine than those fed high Cu levels (160 mg/kg), probably as a compensatory mechanism to maintain Cu homeostasis. Roura et al. [11,25] pointed out that the preference mechanisms of an animal for a feed are mainly determined by chemosensory signals from the upper gastrointestinal tract (mouth and stomach; short term) and by the integration of post-gastric signals (from small or large intestines; long-term) to the brain. Thus, the nutritional value of the food can be distinguished and translated into a physiological stimulus that ends up triggering a greater or lesser consumption of food [11,25]. Previous studies have shown that animals are able to adjust their feed intake based on nutrient requirements, the nutritional value of diets, and health status [6,11]. Concerning minerals, animals deficient in certain minerals such as Na, Ca or P have been found to be able to select a food supplemented with the required nutrient [26,27]. In our laboratory, the same feeding behavior pattern was observed with other nutrients, such as protein. Guzmán-Pino et al. [5] reported that weaned pigs were able to choose feed to correct underfeeding or a protein deficient status and thus re-establish homeostasis. In contrast, Coble et al. [8] reported that finishing pigs had a higher preference for diets without supplementary Cu than those supplemented with 150 mg/Cu kg. It must be noted that unlike weaned pigs, finishing pigs have already overcome the temporary physiological Cu deficit and gastrointestinal stress produced by weaning. Therefore, the preference of an animal for a certain feed may be more than just a matter of flavor perception [28], and probably reflects an interrelationship between the perception of the flavor of the feed and its post-ingestive effects [11]. Among all the positive effects attributed to Cu supplementation, Bikker et al. [7] concluded that approximately 75% of the growth promotor effect of Cu in weaned pigs is explained by the increase in feed intake. The authors also showed that the linear supplementation of Cu (15, 80, 120 and 160 mg/kg) in weaned pig diets resulted in a linear improvement in growth performance. Since in the present study pigs were offered nutritional and high doses of Cu at the same time, no distinctions in growth performance could be established. However, in a previous study performed in our laboratory, the supplementation of weanling pig diets with 160 mg Cu/kg increased ADFI (360 to 379 g), gain to feed ratio and BW(16.6 to 17.7 kg) compared to diets with 15 mg/Cu kg after 42 days of administration ($p < 0.05$) [29].

When Cu sources were contrasted at 150 mg Cu/kg, a large preference was observed for Cu hydroxychloride compared to Cu sulfate. Based on human studies, it is known that the taste of Cu in water depends on its chemical structures and solubility, and the taste has been described as metallic, bitter and bloody [12,13]. Since, a pig's tongue has approximately three times more taste buds involved in the complex process of diet selection compared to humans [14], it is likely that taste perception in pigs is superior to that of humans. Cuppett et al. [13] showed that free Cu ions and soluble Cu complexes in a pH range of from 6.5 to 7.4 can be readily tasted in water. The results of the present in vitro assay showed that Cu hydroxychloride solubility decreased from 100% at pH 2.5 to 9% at pH 6.5 (approximate oral cavity and intestinal pH), unlike Cu sulfate (99–98%, respectively). In agreement with this, Pang and Applegate [30] reported that the order of the solubility of three Cu sources at 250 mg/kg was Cu sulfate > Cu lysine > Cu hydroxychloride for each pH measured (pH 2.5, 5.5, and 6.5). Therefore, it could be speculated that the taste perception of pigs is more intense when Cu sulfate is added compared to Cu hydroxychloride, mainly due to its solubility characteristics. Early studies with finishing pigs reported a higher preference for diets supplemented with Cu at 150 mg/kg as hydroxychloride (65% vs. 35%) than diets with Cu sulfate over 15 days [8]. Likewise, when weaned pigs were offered diets supplemented with Cu at 160 mg/kg as hydroxychloride or sulfate, a strong preference was observed for hydroxychloride compared to sulfate during the first two weeks post-weaning (76% vs. 24% and 81% vs. 19%, respectively) [31].

In the present in vitro assay, Cu solubility was affected by the addition of phytic acid. The largest inhibition of Cu solubility was observed at pH 6.5, that is, Cu sulfate inhibited more intensely than Cu hydroxychloride. Likewise, soluble PP concentrations were affected by the increase in Cu doses and were more critical at pH 6.5 and with the Cu sulfate source. High levels of Cu as sulfate have been described to interfere with phytate, especially at intestinal pH (6.5), resulting in chelate complexes that tend to be resistant to the hydrolytic activity of phytases [13,14]. In 2006, Pang and Applegate [16] in a comparative study reported that adding higher Cu concentrations inhibited PP hydrolysis by phytase at pH 5.5 and 6.5, while Cu as hydroxychloride and lysinate inhibited PP hydrolysis much less than Cu as sulfate pentahydrate, chloride or citrate. Once more, these differences may be the result of the different solubilities among Cu sources.

5. Conclusions

In conclusion, pigs' preference for an essential nutrient like Cu responds to a more complex physiological mechanism than just a flavor perception. Weaned pigs were able to choose diets supplemented with higher levels of Cu, probably due to its systemic post-ingestive effect in order to re-establish Cu homeostasis after weaning. Pigs' Cu taste perception between sources is also attributed to the solubilities and chemical differences of the sources. Pigs showed a greater preference for Cu hydroxychloride diets than those containing the more soluble Cu sulfate. Complementary studies using new approaches, such as genomic tools, should be conducted to explore the biological mechanism behind Cu preference at this stage.

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CHAPTER V

Effects of two zinc supplementation levels and two zinc and copper sources with different solubility characteristics on the growth performance, carcass characteristics and digestibility of growing-finishing pigs

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Effects of two zinc supplementation levels and two zinc and copper sources with different solubility characteristics on the growth performance, carcass characteristics and digestibility of growing-finishing pigs

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Abstract

The present study was conducted to evaluate the effect of two Zn supplemented levels and two Zn and Cu sources (sulphate and hydroxychloride) on growing-finishing pigs. An in vitro study and an in vivo study were conducted. In the in vitro study, Zn solubility from each source at different Zn supplementation levels was evaluated, as well as the phytic phosphorus (PP) solubility derived from the interaction or not with phytic acid at similar conditions to those found in digestive tract. The most critical interaction of Zn with phytic acid was at pH 6.5 and with Zn sulphate, resulting in the reduction in PP solubility. In the in vivo experiment, a total of 444 pigs ([Duroc × Landrace]×Pietrain; initial BW: 18.7 ± 0.20 kg) were allotted to 36 pens in a randomized complete block design (2 × 2) factorial arrangement with two Zn and Cu sources and two Zn supplemental levels (20 and 80 mg/kg). The Cu supplementation was fixed at 15 mg/kg for all diets. There was no effect of the interaction between mineral source × Zn level or Zn level on growth performance or carcass characteristics ($p > .10$). Apparent total digestibility of Zn and Cu along with carcass yield was higher for pigs fed hydroxychloride than pigs fed the sulphate counterparts ($p < .05$). Feeding low levels of Zn decreased Zn (45.5%; $p < .0001$) and Cu(18.5%; $p = .018$) faecal excretion. In conclusion, under commercial conditions, feeding growing-finishing pigs with Zn levels below those established by the European Union regulation did not affect growth performance and carcass characteristics. Reducing dietary mineral (Zn and Cu) diet content resulted in a lower faecal mineral excretion. Pigs fed sulphate minerals had an improved performance during grower period, while pigs fed hydroxychloride minerals showed an improved performance during finishing period and a greater carcass yield and mineral digestibility than those fed sulphates.

KEYWORDS

carcass characteristics, copper and zinc, digestibility, growing pigs, solubility, trace mineral sources

1 | INTRODUCTION

Trace minerals play an essential physiological role for the normal growth and health of animals. For instance, Zn and Cu are involved in the synthesis of more than 300 enzymes and proteins, immune system activation and bone development, among other functions (Suttle, 2010). The National Research Council (NRC) (2012) established a total requirement of Zn (50–60 mg/kg feed) and Cu (3–4 mg/kg feed) for pigs from 25 to 135 kg body weight (BW). European institutions such as FEDNA (2013) and INRA (1989) have also emitted recommendations for the total feed content of Zn (80 and 100 mg/kg feed) and Cu (8 and 10 mg/kg feed) in growing-finishing pig diets. However, in non-European Union (EU) countries (e.g. United States, China, Brazil), the swine industry supplements grower-finisher diets at higher levels than NRC requirements, such as in United States the supplementation is approximately 1.6 times for Zn and 25.8 times for Cu (Flohr et al., 2016), usually to promote growth (Coble et al., 2017; Liu et al., 2018) and prevent intestinal dysbiosis (Højberg, Canibe, Poulsen, Hedemann, & Jensen, 2005; Liu et al., 2018). At high supplemental, and therefore total dietary levels, trace minerals are barely absorbed, while may interact with other nutrients (Pang & Applegate, 2006; Walk, Wilcock, & Magowan, 2015) or with the activity of other additives, such as phytase (Akter, Graham, & Iji, 2015; Augspurger, Spencer, Webel, & Baker, 2004). Moreover, due to the efficient homoeostasis systems, when these minerals are supplemented over the animals' requirements, the mechanisms of absorption are reduced while increasing the endogenous secretions, mainly to intestinal pathways (pancreatic and bile secretions) (Goff, 2018; Spears, 1996; Suttle, 2010). Therefore, large amounts of trace minerals are excreted in manure. Subsequent applications of manure to the soil may increase the ecotoxicity of plants, microorganisms and soil, which is a major environmental concern (EFSA FEEDAP, 2016). Likewise, there is an increasing public health concern due to the association of the great dietary, and therefore environmental, Zn and Cu contents with the increasing microbial resistance rates to antibiotic agents by co-selection, as well as the possibility of transferring of such resistant bacteria from food-producing animals to humans such as farmers, veterinarians and consumers (Ciesinski et al., 2018; Yazdankhah, Rudi, & Bernhoft, 2014). To prevent environmental and public health concerns, dietary trace mineral levels can be reduced without affecting the productive performance of pigs. Therefore, EU countries have established maximum total Zn (120 mg/kg) (European Commission, 2016) and Cu (25 mg/kg) (European Commission, 2018) levels for grower-finisher pigs.

Sulphate mineral sources (35% Zn; 25% Cu) are the most used mineral sources in the animal feed industry due to their high solubility and relatively low cost compared to hydroxychloride and organic bound mineral sources. Sulphates are characterized by having a labile molecular bond that connects the metal ion with the sulphate group, allowing high solubility in water and acid solutions (Guo et al., 2001; Park & Kim, 2016). Therefore, sulphates are frequently used as a reference point for comparing bioavailability of different mineral sources (Guo et al., 2001; Park & Kim, 2016). Alternatively to sulphates, hydroxychloride mineral sources (55% Zn; 54% Cu) have a crystalline structure formed by covalent bonds located between the soluble metal ion, multiple hydroxyl groups and the chloride ions (Zhang & Guo, 2007), with low solubility in water but high solubility in acid solutions (Cao, Henry, Ammerman, Miles, & Littell, 2000). This makes them less reactive with other components in the diet (Lu et al., 2010; Luo et al., 2005). The difference between hydroxychloride mineral sources and chelated or organic counterparts is that the coordinate covalent bond is located between the metal ions and organic molecules (e.g. amino acids, peptides) (AAFCO, 2002).

In the present study, we hypothesized that due to the chemical properties of the trace mineral sources the productive performance of grower-finisher pigs fed hydroxychloride minerals would be higher than those fed sulphates when Zn and Cu doses are lower than those established by the EU regulations. In order to test the hypothesis, a preliminary in vitro assay and a successive in vivo experiment were conducted. To further understand the Zn solubility from each source and the degree of interaction with phytic acid, as the major binding molecule present in swine diets, the in vitro assay was performed at several Zn concentrations (50, 100, 200 and 300 mg Zn/L) from each source and under similar conditions to those found throughout the gastrointestinal tract (pH 2.5, 4.5 and 6.5). Likewise, the phytic phosphorus (PP) solubility was evaluated. An early similar in vitro assay was performed to assess the Cu solubility characteristics from both mineral sources (Villagómez-Estrada, Pérez, van Kuijk, et al., 2020). The in vivo experiment was aimed to evaluate the effect of two supplemented Zn levels (20 mg/kg as a low level and 80 mg/kg as a nutritional level) as well as the effect of two Zn and Cu sources (sulphate and hydroxychloride) on growth performance, carcass characteristics, tissue mineral content and mineral apparent total tract digestibility in grower-finisher pigs reared under commercial conditions. The supplemental level of Cu was fixed at 15 mg/kg for all diets.

2 | MATERIALS AND METHODS

All animal experimentation procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona in compliance with the EU guidelines for the care and use of animals in research (European Parliament, 2010).

2.1 | In vitro solubility test

The methodology for in vitro test is described in an early in vitro test (Villagómez-Estrada, Pérez, van Kuijk, et al., 2020) performed to assess the Cu solubility characteristics from both mineral sources. Briefly, the solubility of Zn sulphate, Zn hydroxychloride and the concentration of soluble PP were measured in duplicate. Zn from each source was added at concentrations of 50 mg/L, 100 mg/L, 200 mg/L and 300 mg/L in 200 mM glycine buffer (pH 2.5) (Merck, Germany) and 200 mM sodium acetate buffer (pH 4.5 and 6.5)

(Merck, Germany). Zn was mixed with 20 ml of buffer with and without 2.9 mM phytate (Merck, Germany), incubated at 41°C in a shaking water bath for 1 hr and filtered through 42-µm Whatman filter paper. The solubility of Zn and PP was analysed with inductively coupled, plasma-optical emission spectroscopy (ICP-OES, model Optima 4300DV, Perkin-Elmer Inc.; Waltham; MA, US). The solubility of PP was expressed as mg/L, whereas the solubility of Zn was calculated using the following equation:

$$\text{Mineral Solubility, \%} = \left[(\text{Soluble mineral}) / (\text{Total mineral})\right] \times 100$$

2.2 | In vivo experiment

2.2.1 | Animals and Housing

The experiment was performed on a commercial farm in Catalonia, Spain, from February to June with a single batch of pigs. At the end of the nursery phase (63 ± 1.1 d of age), 444 pigs ([Duroc × Landrace] ×Pietrain) purchased from a different commercial farm and with an initial average BW of 18.7 ± 0.20 kg were used in a 105-d study. Pigs were weaned at 28 ± 1.1 d of age and nursed during 35 d under commercial conditions and fed the same commercial pre-starter and starter diets. Equal numbers of boars and gilts were ear tag identified and blocked according to sex and initial BW (heavy: 21.7 ± 1.4 kg and light: 15.7 ± 1.9 kg). Pigs were randomly distributed into four experimental diet groups in 36 pens (9 replicate pens/treatment). Pigs were allocated to 12 large pens (10.2 m^2) of 15 animals and 24 smaller pens (7.5 m^2) of 11 animals. Each pen had a fully slatted floor and was equipped with a commercial non-lidded hopper and a nipple drinker to provide ad libitum access to feed and water. The facility was environmentally controlled (temperature and ventilation rate) using thermostatically controlled heaters and exhaust fans.

2.2.2 | Experimental design and dietary treatments

Three-phase diets (Table 1) were formulated to meet or exceed nutrient requirements (NRC, 2012): the pre-grower phase from d 1 to 21, the grower phase from d 21 to 84 and the finisher phase from d 84 to 105. Four experimental diets were prepared according to a 2 × 2 factorial arrangement, with two Zn and Cu sources (sulphate and hydroxychloride) and two Zn supplementation levels (low: 20 mg/kg, and nutritional: 80 mg/kg). The supplementation level of Cu was fixed at 15 mg/kg for all diets. To achieve these supplemental levels, the total amount of Zn sulphate monohydrate (35%; Pintaluba, Reus, Spain) added was 228.6 g/ton in the nutritional diet, and 57.1 g/ton in the low diet. The supplementary rate of Cu sulphate pentahydrate (25%; Pintaluba, Reus, Spain) was 60.0 g/ton for both sulphate diets. The supplementary amount of hydroxychloride Zn (55%, IntelliBond Z; Trouw Nutrition, the Netherlands) was 145.5 g/ton in nutritional diet and 36.7 g/ton in low diet. The supplemental rate of Cu (54%, IntelliBond C, Trouw Nutrition, the Netherlands)

TABLE 1 Composition of the basal diet for the three phases, as fed-basis[a]

Ingredients, %	Pre-growing	Growing	Finishing
Maize	30.00	32.00	32.00
Barley	21.34	13.68	14.65
Wheat	15.01	32.00	32.00
Soya bean meal 47% CP	13.37	12.64	11.99
Extruded soya beans	5.00	-	-
Canola meal	-	5.00	5.00
Carob bean pulp	4.00	-	-
Wheat middlings	4.00	-	-
Sugar beet pulp	2.50	-	-
Lard	1.64	1.77	1.69
DL-Methionine	0.11	0.07	0.04
L-Lysine	0.45	0.49	0.46
L-Threonine	0.16	0.17	0.15
L-Valine	-	0.02	-
L-Tryptophan	-	0.02	0.01
Monocalcium phosphate	0.86	0.24	0.21
Calcium carbonate	0.66	0.97	0.91
Sodium bicarbonate	0.15	0.13	-
Salt	0.35	0.40	0.49
Vitamin-premix nucleous[b]	0.40	0.40	0.40
Calculated composition			
NE, kcal/kg	2,375	2,400	2,400
CP	16.0	15.8	15.5
Ether extract	4.7	4.0	3.9
Ca	0.60	0.65	0.62
Total P	0.56	0.42	0.42
Dig P	0.35	0.26	0.26
Analysed composition			
CP	15.8	15.5	15.5
Ether extract	4.3	3.4	3.6
NDF	13.0	11.9	12.4
Ash	4.6	5.5	4.1
Ca	0.70	0.78	0.66
P	0.59	0.45	0.40

[a] Pre-grower phase diets were fed from d 0 to 21, grower phase diets from d 21 to 84 and the finisher phase diets from d 84 to 105.

[b] Provided per kg of feed: vitamin A (acetate): 7,150 IU; vitamin D3 (cholecalciferol): 1,320 IU; vitamin E: 25 IU; vitamin K3: 1.10 mg; vitamin B1: 0.88 mg; vitamin B2: 2.75 mg; vitamin B6: 1.10 mg; vitamin B12: 0.02 mg; vitamin B3: 16.5 mg; acid D-pantothenic acid: 8.80 mg; biotin: 0.01mg; Fe (sulphate): 60 mg; Mn (oxide): 50 mg;; I: 0.55 mg; Se (sodium): 0.27 mg. Butylhydroxytoluene: 0,09 mg; sepiolite: 3.46 mg; phytase: 500 FTU (Axtra® PHY, Danisco Animal Nutrition, Marlborough, UK); glucanase: 1,500 VU and xylanase: 1,100 VU (Rovabio Excel AP, Adisseo, France).

in both hydroxychloride diets was 27.8 g/ton. Phytase was added at 500 FTU per kg of complete feed (Axtra® PHY, Danisco Animal Nutrition, Marlborough, UK). A vitamin-premix without Zn and Cu was prepared. For each dietary treatment, Zn and Cu products were pre-mixed with 25 kg of basal diet before being incorporated directly into the mixer during the feed manufacturing process. Finisher phase diets were supplemented with titanium dioxide (0.5%) as an indigestible marker. In order to avoid cross-contamination with elements from previous productions, feed was manufactured in an appropriate rank order starting with the lower concentrations to be included in the diet. All diets were offered in mash form. Composite samples (1 kg) were collected during the bagging process and were representative of each experimental treatment. Antibiotics or feed additives with antimicrobial properties were not included in the diets.

2.2.3 | Experimental procedures and sampling

Individual pig weights and feed leftovers were recorded at the end of each feed phase. From these data, average daily gain (ADG), average daily feed intake (ADFI) and gain: feed (G:F) were calculated. In order to reach the market slaughter weight (110 kg), pigs were reared for 25 additional days with the same dietary treatment. During this period, only the individual BW of pigs was recorded one day before slaughter.

At the end of pre-growing phase, one pig per pen was selected based on the mean BW within the pen (median) to take blood samples by jugular puncture. The same pig was sampled at the end of growing and finisher phases and during slaughter process. Blood samples for determining Zn and Cu were collected into 10-mL vacutainer tubes (BD Vacutainer®, Z, BD-Plymouth, UK) that were free of detectable Zn. Serum was obtained after centrifugation (3,000 × *g* for 15 min) and immediately frozen at −20°C. At d 105, faecal samples were collected from three pigs per pen through digital stimulation, pooled and immediately frozen at −20°C. At the slaughterhouse, all animals were electrically stunned and killed by exsanguination. Data on the carcass characteristics were collected using an Autofom (SFK, Herlev, Denmark). During the slaughter process, samples of the medial right lobe of the liver (excluding the gall bladder) and right front toe were collected from the selected pig per pen, after weighing the hot carcass and immediately frozen at −20°C until analysis.

2.2.4 | Chemical analysis

Samples of feed were milled at 0.5 mm before chemical and mineral analysis. Analytical determinations of diets were performed according to the AOAC International, (2005) methods for dry matter (Method 934.01), the Dumas Method for crude protein (Method 968.06), traditional Soxhlet extraction for ether extract (Method 920.39) and ash (Method 942.05). Neutral detergent fibre was analysed using the Ankom nylon bag technique (Ankom 200 fiber Analyzer, Ankom Technology, Macedon, NY).

Liver and faeces were dried in a forced-air oven at 102°C for 12 hr, until constant weight, and then milled at 0.5 mm. Bone ash determination protocol was based on the methodology described by Brenes et al., (2003). Briefly, front toe was dissected, and the IV metacarpal was autoclaved for 30 min at 121°C to remove all the adherent tissues, including cartilage. Subsequently, bone was oven-dried for 12 hr at 102°C and then soaked in acetone under a chemical hood for 48 hr to extract fat. After this period, the bone was again oven-dried for 12 hr at 102°C and weighted before being broken in the middle to be ashed overnight at 550°C in a muffle furnace. Systematically after the incineration process, bone was again weighed, finely ground with mortar, sieved to a particle size of 0.5 mm and homogeneously mixed before mineral analysis. Bone ash content was calculated based on fat-free dried bone, and the mineral content is expressed on bone ash basis.

Prior mineral analysis by ICP-OES samples of feed, faeces, liver and bone ash were digested with concentrated nitric acid (HNO_3, 65%) in a microwave oven (model Ultrawave, Milestone Srl, Sorisole, Italy). Samples of feed and faeces (0.5 g) were digested with 5 ml of HNO_3 and 0.2 ml of hydrofluoric acid (HF; 40%) at 240°C for 30 min. For samples of liver, 0.25 g was digested with 5 ml of HNO_3 at 220°C for 15 min; for bone ash samples, 0.20 g was digested with 4 ml of HNO_3 at 220°C for 15 min. Serum samples (0.25 ml) were diluted in 1:20 volume ratio with 5 ml of a solution constituted by 0.05% ethylenediaminetetraacetic acid (EDTA) and 0.5% of ammonia (NH_3). Immediately after digestion or dilution, samples were analysed by ICP-OES. All samples were analysed for Zn and Cu content.

The coefficient of apparent total tract digestibility (CATTD) of trace minerals was calculated as follow:

$$\mathrm{CATTD} = 1 - \left[(TiD \times NuF) / (TiF \times NuD)\right]$$

where *TiD* and *TiF* represent the concentrations of the non-digestible marker in the diet and faeces, respectively; *NuD* and *NuF* represent the nutrient concentrations in the diet and faeces respectively.

2.3 | Statistical analysis

Data were analysed as a randomized complete block design using the MIXED procedure for mixed linear models of SAS (version 9.4, SAS Institute; Cary, USA). The model included the fixed effects of mineral source, Zn level and their interaction (source × level). Moreover, the block of BW category and sex were considered as random effects. Pen was considered the experimental unit for the performance parameters. The carcass characteristics and mineral tissue content were analysed with individual pig as the experimental unit. The normality and homoscedasticity of data were examined using Shapiro–Wilk test and assessing the normal plot before statistical analysis. When the two-way interaction between mineral source and Zn level was not significant, the interaction was removed from the model and the data re-analysed for main factors. Therefore, the main effects are discussed for responses in which the interaction was not significant. Significantly different means were separated using Tukey adjust. Significance was determined at a probability of $p \le .05$, and tendencies were considered when the p-value was between > 0.05 and < 0.10.

3 | RESULTS

3.1 | In vitro solubility test

The results of Zn solubility sources and their interaction with phytic acid are shown in Figure 1. Overall, the presence of phytic acid decreased the mean Zn solubility as pH increased (from 89% at pH 2.5 to 6% at pH 6.5; Figure 1), regardless of the source. At pH 2.5, the mean solubility of Zn, as an average of the four concentrations, was 100% and 94.7% for Zn hydroxychloride and Zn sulphate respectively (Data not shown). The presence of phytic acid at pH 2.5 reduced the mean solubility of Zn from 97% to 89%, regardless of the source or dose (Data not shown). At pH 4.5, the presence of phytic acid decreased the Zn solubility as the Zn dose increased in both sources (Figure 1c). Phytic acid reduced the Zn hydroxychloride solubility from 100% (without phytic acid) to 43% at pH 4.5 (Figure 1a,c), whereas the mean Zn solubility of sulphate decreased from 98% (without phytic acid) to 55% (Figure 1a,c). At intestinal pH (6.5), the mean solubility of Zn hydroxychloride decreased from 73% to 6% in the presence of phytic acid, while the mean solubility of Zn sulphate decreased from 99% to 6% (Figure 1b,d).

Overall, soluble PP decreased as the pH solution and the Zn dose increased, regardless of the source (pH 2.5 = 424 mg/L; pH 4.5 = 371 mg/L; and pH 6.5 = 312 mg/L; Figure 1c,d). At pH 2.5 and 4.5, the concentration of soluble PP was similar for both sources (Figure 1c). However, at pH 6.5, increasing levels of Zn as sulphate, but not as hydroxychloride, notably reduced the PP soluble content (298 versus 326 mg/L; Figure 1d).

3.2 | In vivo experiment

3.2.1 | Growth performance and carcass characteristics

The analysed mineral content in feed is shown in Table 2. The expected differences between low and nutritional levels of Zn in the diets were achieved with both sulphate and hydroxychloride mineral sources. The Cu content in all diets was within the expected values.

There was no effect of the interaction between mineral source and Zn supplemented level for any of the growth performance responses ($p > .10$). Therefore, data were re-analysed and only the main effects of mineral source and Zn supplemented level are presented in Table 3. The Zn supplementation level was not observed to have any effect on the growth performance during the experimental periods ($p > .10$; Table 3). At the end of the grower period, pigs fed

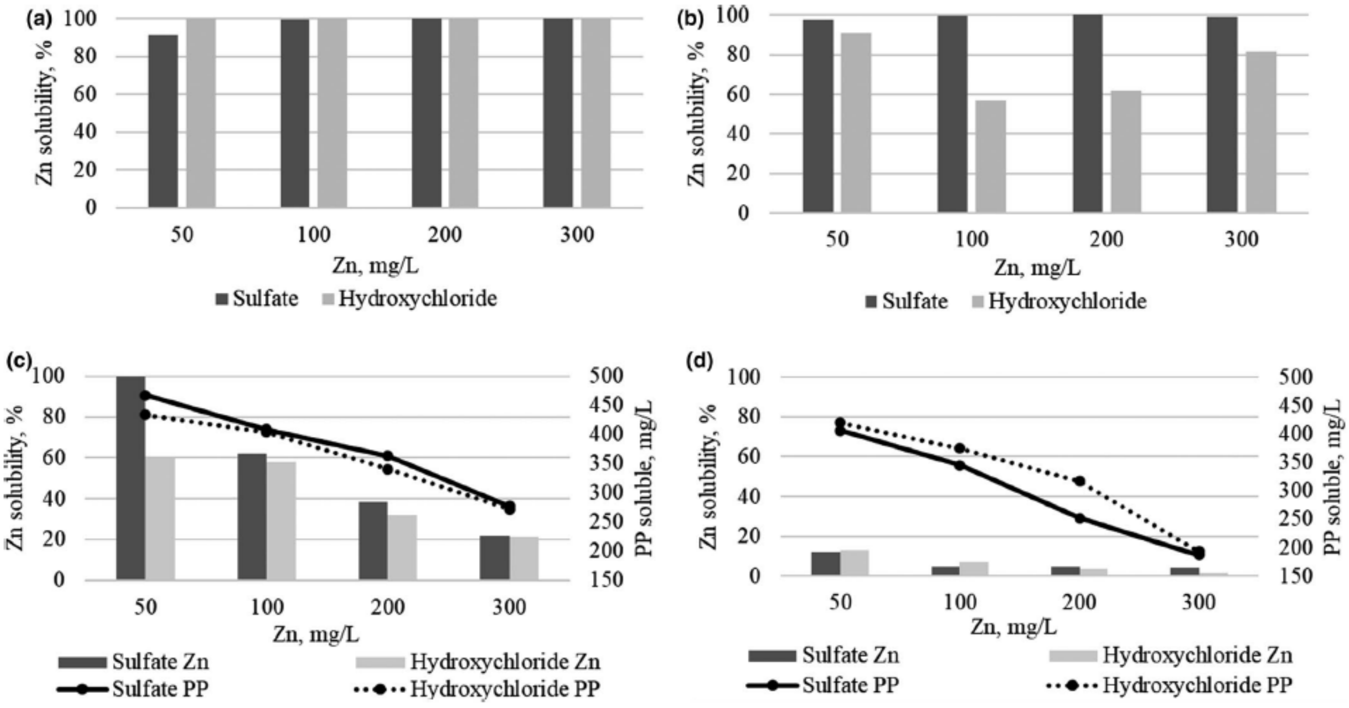

FIGURE 1 Effects of Zn source and level on Zn solubility and soluble phytic phosphorus content at without (a and b) and with (c and d) phytic acid addition at pH 4.5 (a and c) and pH 6.5 (b and d) respectively

TABLE 2 Analysed trace mineral content in experimental diets

Zn and Cu source	Added Zn, mg/kg	Added Cu, mg/kg	Pre-grower, mg/kg		Grower, mg/kg		Finisher, mg/kg	
			Zn	Cu	Zn	Cu	Zn	Cu
Sulphate	20	15	54.1	22.0	73.4	25.4	60.7	17.5
	80	15	116.6	30.8	117.6	19.8	115.2	20.5
Hydroxychloride	20	15	50.7	19.6	90.0	22.7	62.0	15.4
	80	15	109.6	20.0	130.0	21.7	97.6	15.7

Zn and Cu sulphate mineral source had a higher ADG (743 versus 713 g; $p = .006$) and G:F (0.524 versus 0.485; $p = .027$) than those fed Zn and Cu hydroxychloride mineral source. However, at the end of the finisher period pigs fed hydroxychloride mineral source diets tended to have a higher ADG than those fed the sulphate counterparts (774 versus 728 g; $p = .088$). No signs of Zn deficiency, like parakeratosis or decreased growth, were observed. No mineral source × Zn supplemented level interaction was observed for any of the carcass characteristics ($p > .10$; data not shown). Likewise, no effect of dietary Zn level was observed ($p > .10$). Pigs fed Zn and Cu hydroxychloride source had a higher carcass yield percentage ($p < .0001$; Table 3) than those fed Zn and Cu sulphate mineral source.

3.2.2 | Apparent total tract digestibility and faecal mineral excretion

A two-way interaction between mineral source × Zn supplemented level was observed in the CATTD of Zn and Cu ($p < .05$; Figure 2). Pigs fed Zn hydroxychloride at 80 mg/kg had a greater CATTD of Zn (0.39 versus 0.33) and Cu (0.41 versus 0.08) than those fed Zn sulphate at 80 mg/kg, being the nutritional supplemental levels of Zn from both mineral sources intermediate.

A two-way interaction mineral source × Zn supplemented level was also observed for Cu faecal excretion ($p = .011$; Figure 3d). Pigs fed Zn sulphate at nutritional supplemental levels excreted higher quantities of Cu than those fed Zn sulphate at low supplemental levels (159.6 versus 107.1 mg/kg), whereas pigs fed Zn hydroxychloride at both nutritional and low supplemental levels were intermediate (112.1 and 114.4 mg/kg respectively). Feeding diets with lower supplemental levels of Zn decreased the Zn ($p < .0001$) and Cu ($p = .018$) excretion by 45.5% and 18.5% respectively (Figure 3c and 3d).

3.2.3 | Mineral tissue content

There was no effect of the interaction between mineral source × Zn supplemented level in any of the tissue mineral content except for Cu content in liver (Table 4). Pigs fed Zn sulphate at 80 mg/kg had a higher Cu content (92.0 mg/kg) than pigs with the other experimental diets ($p < .0001$; Figure 3b), while no differences in Zn liver content were observed ($p > .10$; Figure 3a). Feeding diets with Zn at 80 mg/kg increased liver and the metacarpal content of Zn ($p < .05$; Table 4). Likewise, increasing dietary levels of Zn increased the Cu content in the serum and liver ($p < .01$). Pigs fed sulphate trace minerals tended to store higher levels of Cu in the liver than those fed hydroxychloride minerals ($p = .090$). All treatments showed low levels (<20 mg/kg) of Cu storage in the metacarpal bone, below the ICP-OES detection limit.

4 | DISCUSSION

4.1 | Growing-finishing pig responses to low dietary Zn levels

The current estimates recommended by European institutions of animal nutrition as well as NRC values for Zn and Cu are considered the minimum requirements for the normal growth of pigs (25–135 kg BW). Contrary to the strategy followed for amino acid, N, Ca and P requirements, which are generated by a modelling approach, the Zn and Cu requirements are derived from a critical evaluation of nutrient requirement studies (NRC, 2012). In accordance with our study, previous studies conducted in growing-finishing pigs have also shown no differences in growth performance and carcass characteristics between growing-finishing pigs fed diets with low or non-supplementary levels of Zn and those fed Zn levels similar or up to NRC requirements (Cemin, Woodworth, et al., 2019; Creech et al., 2004; Gowanlock, Mahan, Jolliff, Moeller, & Hill, 2013; Paboeuf, Nys, & Corlouer, 2000) even under a restricted floor space allowance (Holen, Rambo, Hilbrands, & Johnston, 2018). In general, the requirements for most of the nutrients (as percentage of the diet), including trace minerals, decrease as the BW of the pig increases (NRC, 2012). For instance, higher levels of Zn (100 mg/kg feed) and Cu (6 mg/kg feed) are needed for pigs from 5 to 11 kg BW compared to growing pigs (NRC, 2012). The lack of growth response between these two supplemented levels of Zn in the present study could be attributed to (a) the absence of intestinal and immunological challenges experienced by growing-finishing pigs. Unlike newly weaned pigs, growing-finishing pigs have a mature immune system and intestinal microbiome (Wang et al., 2019), which is already adapted to solid feed and other stress factors such as the environmental conditions. (b) The greater intake capacity of growing-finishing pigs may allow satisfying their Zn requirement as more feed is consumed, although the bioavailability of natural minerals in the cereals and grains of their diet is low or not completely known. (c) The addition of phytase (in the present study

TABLE 3 Growth performance and carcass characteristics of pigs fed diets with two Zn and Cu sources (sulphate and hydroxychloride) at two Zn levels (20 and 80 mg/kg)

		Growth performance[a]										Carcass characteristics[b]		
		BW, kg				ADG, g				ADFI, g	G:F			
						d								
Zn and Cu Source	Zn level, mg/kg	d 0	d 21	d 84	d 105	d 0–21	22–84	d 85–105	d 0–105	d 0–105	d 0–105	CCW, kg	Lean meat, %	Carcass yield, %
Sulphate		18.87	31.80	78.59	94.06	614.8	743.0	728.4	716.1	1687.1	0.428	74.76	67.13	70.04
Hydroxychloride		18.62	31.44	76.40	92.89	609.9	712.6	773.7	707.2	1734.6	0.409	74.02	67.08	70.57
SEM		3.051	3.655	4.584	5.036	29.52	20.61	31.26	21.67	49.40	0.0083	2.022	0.351	0.232
	20	18.66	31.63	77.81	93.91	617.3	730.5	758.2	716.5	1735.2	0.415	73.96	67.26	70.22
	80	18.83	31.61	77.18	93.03	607.4	725.2	743.9	706.8	1686.5	0.421	74.82	66.96	70.40
SEM		3.049	3.653	4.579	5.032	29.37	20.54	30.95	21.63	48.72	0.0081	2.039	0.359	0.237
p-value														
Source		0.696	0.645	0.086	0.319	0.784	0.006	0.088	0.251	0.325	0.108	0.199	0.816	<0.0001
Level		0.790	0.978	0.608	0.448	0.575	0.611	0.579	0.206	0.312	0.607	0.138	0.165	0.106
Source × Level		0.750	0.841	0.828	0.326	0.363	0.745	0.443	0.281	0.629	0.714	0.479	0.725	0.158

[a] Data are the average value of $n = 9$ replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 18 replicate pens.

[b] Data are the average value of $n = 444$ pigs for the two-way interaction, whereas for the main effects of source and level are the average value of $n = 222$ pigs. CCW = cold carcass weight.

at 500 FTU) may have increased Zn bioavailability and minimized other dietary antagonisms of phytic acid with other minerals and digestive enzymes (Revy, Jondreville, Dourmad, & Nys, 2004; Schlegel, Sauvant, & Jondreville, 2013). (d) The supplementation of high-fibre diets with carbohydrase enzymes (i.e. glucanase and xylanase) may have enhanced the nutrient digestibility, improved intestinal barrier integrity and modulated the intestinal microbiota, consequently, supporting growth performance (Torres-Pitarch et al., 2020). (e) The complex homoeostasis body system of Zn and Cu may also play an important role in supporting the growth performance of pigs, particularly in those supplemented with a low Zn level, mobilizing minerals from the storage sites in case of being necessary (Suttle, 2010). Nevertheless, it is known that pigs fed with Zn-deficient diets usually manifest several subclinical and clinical signs such as decreased water and feed intake, growth depression, parakeratosis, hair loss and discoloration. Among these, parakeratosis is the pathognomonic clinical sign and may mark the end point in response to a long-term insufficient supply of Zn (Brugger & Windisch, 2016). Since in the current study, none of the pigs showed signs of Zn deficiency, and no differences were observed in the long-term growth performance between Zn dietary levels, it could be suggested that feeding pigs with these low levels did not cause a mineral-deficit challenge for the animals.

It is not easy to assess the essential trace mineral status of animals, such as Zn and Cu, because the minerals are involved in many physiological functions and their metabolism is complex and not totally understood (Suttle, 2010). Once absorbed Cu from intestine is primarily stored in liver and thereafter distributed, bind to ceruloplasmin, to other tissues such as kidneys, brain, skeleton and skeletal muscle, but in less proportion (Linder et al., 1998; Roberts & Sarkar, 2008). Therefore, liver is considered the main organ responsible for Cu homoeostasis (Goff, 2018). The low Cu values detected in metacarpal bone in the present study may be attributed to the predilection of body for storing greater amounts of Cu in liver than in bone. Generally, the inclusion of pharmacological doses of Zn has been associated with a decrease in Cu bioavailability (Revy et al., 2004). It is likely that, in response to a dietary Zn overload, more metallothionein (MT) proteins are expressed in the enterocytes, which instead bind Cu, reducing its availability to be absorbed (Goff, 2018; Revy et al., 2004). Others have been proposed a competition between Zn and Cu for the use of the divalent metal transporter 1 (DMT1) (Lutsenko, Barnes, Bartee, & Dmitriev, 2007); however, it is also known that both Zn and Cu have their own specific transporters, which are considered the main pathways used to cross the apical membrane (Goff, 2018). Since in the present study, the exposure level of Zn was within the nutritional range, the Cu long-term storage was not impaired but instead improved as pigs were fed nutritional levels of Zn, suggesting a greater mineral absorption as previously reported (Carlson et al., 2004). Regarding Zn storage in body, as expected, increasing Zn levels in the diets resulted in higher Zn content in target tissues such as liver and bone. The main storages sites of Zn are the liver, muscle and bone (Revy, Jondreville, Dourmad, & Nys, 2003). However, it should be considered that

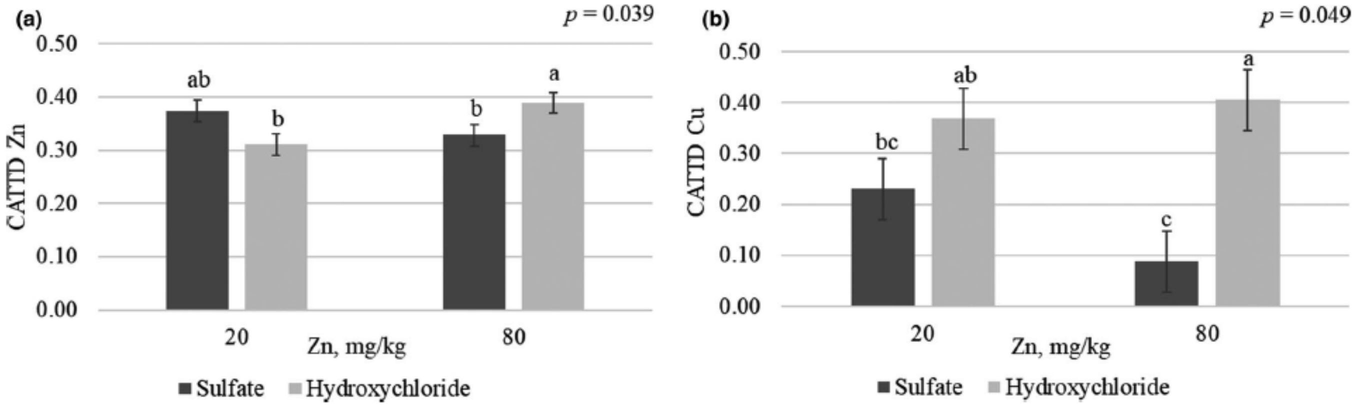

FIGURE 2 Coefficient of apparent total tract digestibility (CATTD) of Zn (a) and Cu (b) at the end of finisher phase (d 105) of pigs fed diets with two Zn and Cu sources (sulphate and hydroxychloride) at two Zn levels (20 and 80 mg/kg). Data are means of 9 replicate pens for the two-way interaction (3 pigs per replicate pen were sampled and pooled). a-c: values with different letters differ significantly for the two-way source × level ($p < .05$)

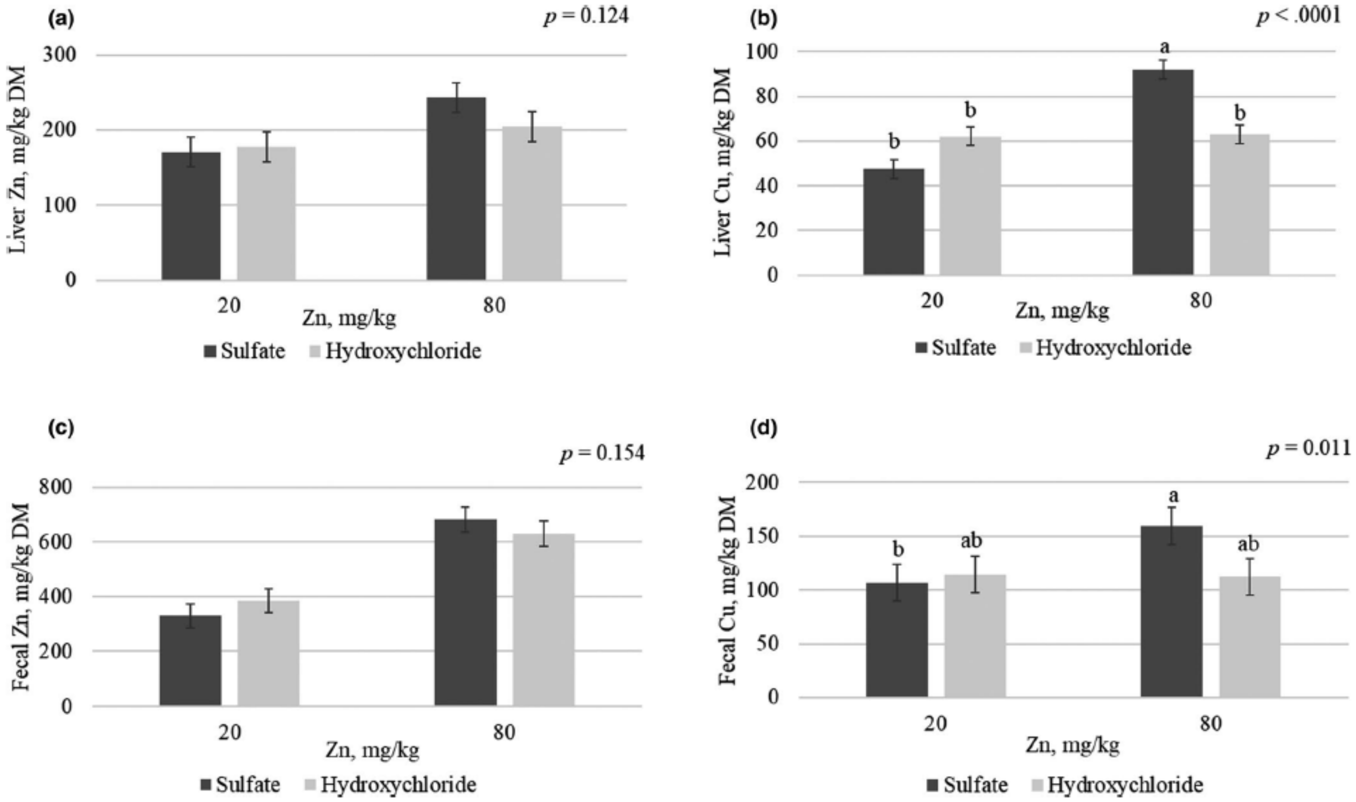

FIGURE 3 Liver trace mineral accumulation (a-b) and faecal excretion (c-d) of pigs fed diets with two Zn and Cu sources (sulphate and hydroxychloride) at two Zn levels (20 and 80 mg/kg). Data are means of 9 replicate pens for the two-way interaction (1 pig for liver and 3 pooled pig for faeces were sampled per replicate pen). a-b: values with different letters differ significantly for the two-way source × level ($p < .05$)

under dietary Zn deficiency the Zn stored in bone cannot be easily mobilized (McDowell, 2003), contrary to others internal pools as liver (Blaabjerg & Poulsen, 2017); therefore, a suitable content of dietary Zn is necessary. In the present study, although pigs fed a low Zn level (20 versus 80 mg/kg) showed lower Zn content in the liver, no differences were observed in growth performance between dietary levels. Nonetheless, complementary studies using additional biomarkers should be considered to confirm that these low dietary supplies match the pigs' metabolic requirements, especially under stressing or poor sanitary conditions in which the Zn requirements for optimizing the immune function may be higher than those for growth (Klasing, 2001).

Reducing dietary Zn levels resulted in a reduction in Zn and Cu faecal excretion of 45.5% and 18.5% respectively. In agreement with

TABLE 4 Tissue mineral content of pigs fed diets with two Zn and Cu sources (sulphate and hydroxychloride) at two Zn levels (20 and 80 mg/kg)

		Trace mineral content[a]				
		Zn			Cu	
Zn and Cu Source	Zn level, mg/kg	Serum, mg/L (d 105)	Liver, mg/kg DM	Bone[b], mg/kg	Serum, mg/L (d 105)	Liver, mg/kg DM
Sulphate		1.07	207.0	219.0	2.02	69.79
Hydroxychloride		1.14	190.7	213.9	1.84	62.50
SEM		0.064	16.53	7.83	0.136	2.947
	20	1.10	173.9	204.9	1.84	54.79
	80	1.10	223.8	227.9	2.05	77.50
SEM		0.065	16.30	7.62	0.136	2.947
p-value						
Source		0.102	0.226	0.635	0.600	0.090
Level		0.977	0.002	0.038	0.010	<0.0001
Source × Level		0.241	0.124	0.912	0.317	<0.0001

[a] Data are of the average values of *n* = 9 replicate pens for the two-way interaction, whereas for the main effects of source and level are the average values of *n* = 18 replicate pens (1 pig per pen was sampled).

[b] Bone ash basis. Cu-detected values are lower than 0.02 mg/g by ICP-OES.

our results, previous studies with pigs have reported that Zn and Cu excretion could be reduced without negative effects on growth performance, for instance, by 50% by reducing micro-mineral supplementation from commercially utilized levels (Zn 150–100 and Cu 25–15 mg/kg, respectively) to 25 and 5 mg/kg respectively (Creech et al., 2004; Liu et al., 2016; Paboeuf et al., 2000; Van Heugten, O'Quinn, Funderburke, Flowers, & Spears, 2004). The absorption and excretion of Zn and Cu are strictly regulated through homoeostatic control mechanisms, and when these minerals are supplemented over the animals' requirements, the excess is not absorbed or is endogenously secreted (Goff, 2018; Spears, 1996), ending up in manure. There are increasing environmental and public health concerns about the swine industry due to its contribution to contaminating soils and ground water with high Zn and Cu levels (Wuana & Okieimen, 2011) and possibly spreading antibiotic resistance by co-selection (Yazdankhah et al., 2014). Therefore, feeding trace minerals to animals as precisely as possible is a potential approach for reducing these environmental and public health risks. The present study found that feeding growing pigs with Zn levels under those established by the EU regulation did not affect growth performance and carcass characteristics, and also resulted in lower faecal mineral (Zn and Cu) excretion.

4.2 | Growing-finishing pig responses to different Zn and Cu sources

Interestingly, at the end of the grower feed period, pigs fed diets supplemented with Zn and Cu as sulphate mineral source had higher growth performance than those fed diets with the hydroxychloride counterparts. The opposed was observed in the finisher feed period, where pigs fed hydroxychloride mineral sources diets showed an improved performance than those fed the sulphate sources. Similar effects were described in a meta-analysis performed by van Kuijk, Jacobs, Smits, and Han, (2019). The authors reported that the supplementation of growing-finishing pig diets with Zn hydroxychloride (80 mg/kg) resulted in a 3.9% improvement in G:F and ADG, during the last feed phase, compared to Zn sulphate source. It is likely that these improvements, during the last period of pig growth, might be related to improvements in carcass characteristics, as a greater carcass yield in the present study. In agreement, results from previous studies reported that the supplementation of growing-finishing pig diets with Zn hydroxychloride (50, 100 and 150 mg/Zn kg) promoted greater hot carcass weight ($p = .041$) (Carpenter et al., 2016), higher carcass yield ($p = .017$) and a tendency for greater hot carcass weight ($p = .058$) (Cemin, Carpenter, et al., 2019) compared with the Zn sulphate supplementation. Likewise, the supplementation of Zn (80 mg/kg) as hydroxychloride mineral source resulted in a higher lean meat percentage ($p = .001$) compared with Zn sulphate supplementation (van Kuijk et al., 2019). Similar improvements on carcass composition were reported in a broiler chicken study with the supplementation of Zn and Cu hydroxychloride (Olukosi, van Kuijk, & Han, 2018). One proposed mechanism of action of hydroxychloride minerals is the influence in the ratio of protein to fat deposition (van Kuijk et al., 2019). Nevertheless, Coble et al., (2017) reported no difference in carcass characteristics when pig diets were supplemented with 75 or 150 mg Cu/kg from a sulphate or hydroxychloride mineral source.

Although the metabolic action of Zn and Cu from different sources is not completely understood, results from previous studies reported different beneficial effects of hydroxychloride minerals on animal health, which may influence the beneficial productive results. For instance, the supplementation of weanling pig diets with Cu hydroxychloride decreased the oxidative stress in the duodenum (Fry et al., 2012; Huang et al., 2015) and increased the serum activity of SOD and ceruloplasmin (Zheng et al., 2018) compared to Cu sulphate. Likewise, the supplementation of Cu and Zn hydroxychloride tended to increase serum GPx activity in weaned pigs (Villagómez-Estrada, Pérez, Darwich, et al., 2020). Although the absorption and retention rates of trace mineral sources were not measured, a greater apparent total tract digestibility was observed for Zn and Cu hydroxychloride, suggesting a better absorption, and therefore less faecal excretion, compared to sulphates. The last could also be related to the solubility characteristics observed throughout the in vitro study and the improvements observed at the end of finisher phase and in carcass characteristics, despite their lower growth performance during grower period.

The results of the present in vitro study of Zn, as well as our previous in vitro study of Cu (Villagómez-Estrada, Pérez, van Kuijk, et al., 2020), reported that sulphates have a greater range of solubility than hydroxychloride, depending on pH, making them more prone to interact with other components of the diet such as phytic acid. Indeed, the mean Cu solubility for sulphate mineral source reported was of 98% in the whole range of pH levels (from 2.5 to 6.5), whereas the hydroxychloride Cu mean solubility decreased drastically from 100% at pH 2.5 and 4.5 to 9% at pH 6.5. The addition of phytic acid resulted in a decrease in Cu solubility of both sources, being most critical at pH 6.5 and with Cu sulphate than with Cu hydroxychloride, which also resulted in a decreased solubility of PP (Villagómez-Estrada, Pérez, van Kuijk, et al., 2020), similar to that observed in the present Zn in vitro study. In this sense, different studies of bioavailability in pigs and broiler chickens have reported a slightly higher bioavailability of Zn hydroxychloride (111%) compared with Zn sulphate (Batal et al., 2001) but greater than Zn oxide (159.3%) (Mavromichalis, Webel, Parr, & Baker, 2001; Zhang & Guo, 2007). Similar relative bioavailability of Cu hydroxychloride compared with Cu sulphate has been reported in pigs (112%) and poultry (109%) (Luo et al., 2005; Miles, O'Keefe, Henry, Ammerman, & Luo, 1998). Nevertheless, in the present study no differences between mineral sources in Zn and Cu tissue content were observed, except for the Cu content in liver. Certainly, the mechanisms of the influence on metabolism of Zn and Cu of both trace mineral sources need further research, especially because after a long-term supplementation of Cu (15 mg/kg), pigs fed Zn sulphate at nutritional levels had a higher Cu content in the liver than the other experimental groups and even higher than the normal range (19 to 56 mg/kg; Puls, 1994). Moreover, besides this greater liver Cu accumulation, a reduced Cu apparent digestibility and a higher Cu faecal excretion were also observed in those pigs. The complex homoeostasis process of Cu is aimed to ensure a constant and sufficient supply of the micronutrient while simultaneously avoiding excess body levels (Scheiber, Dringen, & Mercer, 2013). In this sense, it is possible that to counteract the excessive accumulation of Cu in the liver in pigs fed with a mineral source of Cu sulphate at nutritional levels of Zn, the homoeostasis mechanism reduced the absorption of Cu from the intestine by increasing the sequestration of Cu in MT, while increasing hepatobiliary excretion through the bile into the intestine and, consequently, in the faeces (Bremner, 1998; Gaetke & Chow, 2003). It should be noted that the Cu excreted into bile is poorly available for its reabsorption (Roberts & Sarkar, 2008). Further comprehensive studies involving several complementary analyses need to be performed to elucidate the action mechanisms for hydroxychloride minerals and to understand the trace mineral metabolism better.

In conclusion, feeding growing-finishing pigs with Zn levels below those established by the European Union regulation did not affect growth performance and carcass characteristics in the present conditions. Moreover, reducing dietary mineral (Zn and Cu) content resulted in a lower faecal mineral excretion, which makes it one of the possible approaches for reducing the associated environmental and public health risks. The supplementation of pig diets with hydroxychloride minerals resulted in higher apparent digestibility of Zn and Cu and carcass yield compared with diets supplemented with the sulphate counterparts. Further studies with complementary physiological biomarkers are necessary to confirm that these low dietary levels match the pigs' metabolic requirements.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ANIMAL WELFARE STATEMENT

The authors confirm that they have followed European Union standards for the protection of animals used for scientific purposes (European Parliament, 2010).

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CHAPTER VI

Strategies of inorganic and organic trace mineral supplementation in gestating hyperprolific sow diets: effects on the offspring performance and fetal programming

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NON RUMINANT NUTRITION

Strategies of inorganic and organic trace mineral supplementation in gestating hyperprolific sow diets: effects on the offspring performance and fetal programming

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Abstract

The aim of the present study was to evaluate the effect of trace mineral nutrition on sow performance, mineral content, and intestinal gene expression of neonate piglets when inorganic mineral sources (**ITM**) were partially replaced by their organic mineral (**OTM**) counterparts. At 35 d postmating, under commercial conditions, a total of 240 hyperprolific multiparous sows were allocated into three experimental diets: 1) ITM: with Zn, Cu, and Mn at 80, 15, and 60 mg/kg, respectively; 2) partial replacement trace mineral source (**Replace**): with a 30 % replacement of ITM by OTM, resulting in ITM + OTM supplementation of Zn (56 + 24 mg/kg), Cu (10.5 + 4.5 mg/kg), and Mn (42 + 18 mg/kg); and 3) Reduce and replace mineral source (**R&R**): reducing a 50% of the ITM source of Zn (40 + 24 mg/kg), Cu (7.5 + 4.5 mg/kg), and Mn (30 + 18 mg/kg). At farrowing, 40 piglets were selected, based on birth weight (light: <800 g, and average: >1,200 g), for sampling. Since the present study aimed to reflect results under commercial conditions, it was difficult to get an equal parity number between the experimental diets. Overall, no differences between experimental diets on sow reproductive performance were observed. Light piglets had a lower mineral content ($P < 0.05$) and a downregulation of several genes ($P < 0.10$) involved in physiological functions compared with their average littermates. Neonate piglets born from Replace sows had an upregulation of genes involved in functions like immunity and gut barrier, compared with those born from ITM sows ($P < 0.10$), particularly in light piglets. In conclusion, the partial replacement of ITM by their OTM counterparts represents an alternative to the totally inorganic supplementation with improvements on neonate piglet gene expression, particularly in the smallest piglets of the litter. The lower trace mineral storage together with the greater downregulation of gut health genes exposed the immaturity and vulnerability of small piglets.

Key words: fetal programming, gestational nutrition, small piglets, sow nutrition, trace minerals

Abbreviations

ANPEP	aminopeptidase-N
BW	body weight
CP	crude protein
FDR	false discovery rate
MDA	malondialdehyde
NDF	neutral detergent fiber
NE	net energy
OTM	organic trace minerals
PCR	polymerase chain reaction
TM	trace minerals

Introduction

Although swine genetic selection has successfully increased the litter size (>16 piglets), this has also involved a lower average piglet birth weight, a higher variation of birth weights within the litter, and an increase in the percentage of piglets with low birth weights (even lower than 800 g; Kemp et al., 2018; Oliviero et al., 2019). In general, the presence of small piglets, weighing less than 1 kg, in large litters can range between 15.2% and 25% (De Vos et al., 2014; Feldpausch et al., 2019), who also has a 5.9 times higher risk of dying before weaning compared with their heavier littermates (Feldpausch et al., 2019). Moreover, up to 30% of these small piglets can be exposed to different degrees of intrauterine growth restriction (Foxcroft et al., 2006; Amdi et al., 2013). Birth weight is not only a crucial economic feature in swine production, it is also critical for the piglet development. Indeed, the small vulnerable piglets often show an impaired performance due to a delayed and reduced colostrum intake, and poor thermoregulatory capacity (Kemp et al., 2018), while it may have permanent negative impacts on organ structure and postnatal growth efficiency (Ji et al., 2017) compared with their heavier littermates. Some nutritional interventions during pregnancy, such as the supplementation of diets with functional amino acids (Mateo et al., 2007; Nuntapaitoon et al., 2018) have been proposed to mitigate the incidence of small piglets. Along this line, it is well known that trace minerals (**TM**) are essential nutrients due to their vital roles in a wide variety of physiological processes, including structural and metabolic functions (Suttle, 2010). For instance, in sows, they are critical for metabolism, and growth of fetuses, as well as for the production of colostrum and milk and uterine involution (Hostetler et al., 2003; Suttle, 2010; NRC, 2012). However, unlike energy and amino acid requirements, very little research is available that focuses on the effects and requirements of TM in highly prolific sows, and definitive information is scarce (NRC, 2012). The National Research council (NRC, 2012) estimated a requirement of Zn (100 mg/kg), Cu (10 mg/kg), and Mn (25 mg/kg) for gestating sows. In the swine industry, TM are supplemented at different levels (Zn: 80 to 125 mg/kg; Cu: 6 to 20 mg/kg; Mn: 40 to 60 mg/kg), and frequently with inorganic sources (**ITM**), most as sulfates and oxides. Inorganic sources, as sulfates, are characterized by an unstable structure, making them highly soluble in water and acid solutions, while promoting a negative interaction with other feed components like phytic acid (Villagómez-Estrada et al., 2020) and minerals (Walk et al., 2015). In contrast, organic TM (**OTM**) have a stable chemical structure formed by coordinate covalent bonding between the metal ions and organic molecules (AAFCO, 2021), which make them less prone to feed interactions and therefore to have greater mineral bioavailability (Acda and Chae, 2002). In the present study, the OTM utilized are chelates of protein hydrolysate. Studies conducted in weaned and growing pigs have shown similar (Hill et al., 2014; Liu et al., 2016) or greater efficiency (Veum et al., 2004; Zhao et al., 2014) in promoting growth when ITM were totally or partially replaced by OTM counterparts. However, little is known about the effects of these strategies on the performance of gestating sows (Peters and Mahan, 2008; Ma et al., 2020; Tsai et al., 2020) and, in particular, their likely effects on fetal programming of small piglets. During gestation, after 35 d postmating, when the embryonic implantation has already been established and, consequently, the potential size of the litter, the uterine capacity begins to be the main limiting factor in fetal development, which in terms of nutrition means an increased fetal competition for available maternal nutrients to grow and survive (Langendijk and Plush, 2019). An optimal maternal nutrition, with specific nutrients as TM, may improve the development and survival of fetus within the litter. Consequently, in the present study, we hypothesized that, due to the chemical properties of TM sources, the productive performance of hyperprolific sows fed from 35 d postmating with two combinations and doses of inorganic and organic mineral sources of Zn, Cu, and Mn would be higher than those fed only with a complete dose of inorganic sources. The objectives of the study were to evaluate the effect of a partial substitution of Zn, Cu, and Mn inorganic sources by Zn, Cu, and Mn organic forms, as well as to evaluate the effect of a dietary reduction level on the hyperprolific sow performance, mineral content in colostrum and piglet tissues, as well as intestinal gene expression of neonate piglets. The influence of maternal nutrition on the offspring was evaluated on two neonate littermates from 10 sows per diet, which were selected based on their birth weights (light: <800 g and average: >1,200 g).

Materials and Methods

All animal experimentation procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona (CEEAH 3817) in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

Animals and housing

The experiment was carried out on a commercial farm under Spanish commercial conditions. In Spain, the sow population of hyperprolific line is about 35% of the total sow population. At the end of early gestation (35 d postmating; confirmed gestation by ultrasound), a total of 240 healthy sows from hyperprolific line (DanBred hybrid line; Landrace × Yorkshire, parity 4.5 ± 1.8), obtained from the same commercial farm, were randomly assigned to three experimental diets ($n = 80$). For each experimental diet, sows were individually weighted and housed in two gestation pens, one corresponding to light weight sows with parities from second to fourth and another pen for heavy weight sows with parities from fifth to eighth. Given that from day 35 after mating, there is an intensive sibling competition for maternal nutrients, that conditioned the fetal development, the present experiment was designed to assess how gestational TM nutrition can improve litter development and survival. Therefore, experimental diets were fed daily from day 35 postmating until sows were transferred to farrowing crates (day 110). Each gestation barn (6.7 × 14.2 m; 40 sows) had an electronic feeding station (model Intec-Mac, Mannebeck, Schuttorf, Germany) used to control individual sow feed intake through an electronic ear tag. Feed was provided throughout the day. Sows were fed a mean of 2.4 kg/d up to 35 d postmating, and subsequently a mean of 2.3 kg/d until day 110 postmating, with individual adjustments if necessary. During the experiment, two evaluations were performed at 63 d postmating and at 93 d postmating aimed to adjust individual feed intake of sows using a visual body condition score on a scale from 1 (thin) to 5 (overconditioned) performed by a trained technician (Fitzgerald et al., 2009), and also evaluating the sow back-fat thickness using a veterinary ultrasound scanner (model WED-3000 V, Welld, Shenzhen, China). Thus, sows considered as thin (body condition score of 2 and back-fat thickness between 9 and 13 mm) received an additional 30% of feed only for 30 consecutive days, which resulted on 2.6 kg of average provided feed per day during gestation. Although overweight or fat sows (body condition scores up above 4 and back-fat thickness up to 26 mm) were restricted to 1.9 kg/d resulting in an average provided feed of 2.1 kg/d gestation. The general health and welfare status of sows was daily assessed. Water was provided ad libitum through commercial nipple waterers. Light was provided by daylight (via windows) and artificial light (programmable; from 07 00 h to 19 00 h). The temperature inside the buildings was automatically controlled and maintained in a range between 19 °C and 20 °C using force-speed fans linked to temperature sensors with cooling. At day 110 of gestation, sows were individually weighed and moved to farrowing crates (0.7 × 2.2 m) where the respective experimental diet of lactation phase was provided ad libitum. The farrowing facility was environmentally controlled with 32 individual stalls of fully slatted floor for sows and a heated hard plastic floor for piglets, and equipped with a feeding ball system (Rotecna, Agramunt, Lleida, Spain) and two nipple waterers (1 for sow and 1 for piglets) to ensure feed and water ad libitum. The feeders were manually filled twice a day (08:00 and 15:00 h) to ensure ad libitum intake of the experimental lactation diets.

Table 1. Ingredient and nutrient composition of the basal sow diet, as fed-basis

Ingredients, %	Gestation
Wheat	35.00
Barley	23.50
Wheat bran	20.00
Sunflower cake	10.00
Maize	7.70
Lard	0.50
Lysine 50	0.44
L-Threonine	0.12
Choline chloride 50	0.04
Salt	0.40
Calcium carbonate	1.45
Di calcium phosphate	0.45
Vit–min premix[1]	0.40
Calculated composition	
NE[2], kcal/kg	2,260
CP[3], %	13.01
Ether extract, %	3.17
Crude fiber, %	5.87
Ca, %	0.81
Total P, %	0.56
Dig P, %	0.35
Analyzed nutrient composition, %	
CP	13.03
Ether extract	3.57
NDF[4]	13.27
Ash	6.20
Ca	0.89
P	0.61

[1] The following amounts were provided per kg diet: 10,000 IU vitamin A (acetate); 2,000 IU 25-hydroxy vitamin D3 (HyD, DSM Nutritional Products, Basel, Switzerland); 70 mg DL-alfatocoferol; 1 mg vitamin B1; 2.7 mg vitamin B2; 1.8 mg vitamin B6; 0.03 mg vitamin B12; 11 mg D-pantothenic acid; 15 mg niacin; 1 mg folic acid; 150 mg iron ($FeSO_4$); 0.5 mg iodine ($Ca(IO_3)_2$); 0.4 mg selenium (Na_2SeO_3); 25 mg butylhydroxytoluene.

[2] NE = net energy.

[3] CP = crude protein.

[4] NDF = neutral detergent fiber.

Experimental design and dietary treatments

Gestation basal diet (Table 1) was formulated to meet or exceed nutrient requirements (NRC, 2012). A basal vitamin-premix without Zn, Cu, and Mn was previously prepared. The sources and doses of Zn, Cu, and Mn were subsequently added to this basal vitamin-premix according to the experimental design. Therefore, three vitamin-premix were obtained and consequently added to the basal diet to obtain three different experimental diets. The experimental diets were designed to provide mineral levels close to the swine industry and NRC (2012) requirements, as follows: 1) inorganic trace mineral diet (**ITM**) was supplemented with Zn, Cu, and Mn at 80, 15, and 60 mg/kg, respectively; 2) partial replacement diet (**Replace**) replaced a 30% of ITM diet levels by organic minerals, resulting in a ITM + OTM supplementation of Zn (56 + 24 mg/kg), Cu (10.5 + 4.5 mg/kg), and Mn (42 + 18 mg/kg); and 3) reduce and replace diet (**R&R**) consisted of a 50% reduction of inorganic mineral sources from ITM diet but maintaining the same amount of organic minerals added in Replace diet, thus resulting in a supplementation of Zn (40 + 24 mg/kg), Cu (7.5 + 4.5 mg/kg), and Mn (30 + 18 mg/kg). The inorganic sources of Zn (zinc sulfate monohydrated, 35%), Cu (copper sulfate pentahydrate, 25%), and Mn (manganese oxide, 60%) were purchased from Pintaluba, Reus, Spain. The organic sources of Cu (Optimin Cu, 15%), Zn (Optimin Zn, 15%), and Mn (Optimin Mn, 15%) were provided by Trouw Nutrition, Amersfoort, the Netherlands. Phytase (Ronozyme NP (M), DSM Nutritional Products, Basel, Switzerland) was added at 1,500 FYT per kg of complete feed in all diets. In order to avoid cross contamination with elements from previous productions, feed was manufactured in an appropriate rank order starting with the lower concentrations to be included in the diet. Gestating and lactating diets were offered in pellet form, and the first and last 100 kg of the final diet from each experimental diet were discarded to reduce cross contamination. Experimental diets were prepared in four batches of 4,000 kg each one. From each manufacturing batch, composite samples (1 kg) were collected during the bagging process to be representative of every single experimental diet. Batch samples, from each experimental diet, were mixed and thereafter proportionally split using a riffle splitter into four 250-g samples that were stored for further analysis. Mineral content in feed samples were measured in duplicate.

Experimental procedures and sampling

Sow body weight (**BW**) and back-fat thickness were recorded at days 35 and 110 of gestation. Back-fat thickness was recorded using a portable veterinary ultrasound scanner (model WED-3000 V, Welld, Shenzhen, China) measured at P2 (7.5 cm from midline at last rib). Individual feed intake was recorded daily, according to the electronic feeding station, during the gestation phase under study (35 to 110 d of gestation). Since it was not aimed to evaluate nursery sow performance, the feed intake was recorded until the sows were transferred to farrowing crates. Sow reproductive performance included total number of piglets born as live and stillborn. Only litter weight of alive piglets was recorded. A subset of 20 sows per diet from third to fifth parity was selected for sampling. At day 110, blood samples for mineral analysis were collected by jugular puncture into 10-mL vacutainer tubes (BD Vacutainer, Z, BD-Plymouth, UK) free of detectable Zn. Serum was obtained after centrifugation (3,000 × *g* for 15 min) and immediately frozen at −20 °C. Samples of colostrum (40 mL) were collected from the same sows by hand stimulation of all functional mammary glands within 12 h after farrowing. Colostrum samples were immediately frozen at −20 °C until mineral analysis. At farrowing, two littermates from 10 sows (from the same subset) per experimental diet were selected to take samples. Piglets were selected using their birth BW as a criterion, adjusted from the methodology of Wang et al. (2016) and Bauer et al. (2000), in two categories: light and average littermates. Briefly, a light piglet was defined as having a birth weight between 600 and 800 g and belonging to the lower quartile of litter birth BW, whereas an average littermate had a birth BW within the average birth BW of the litter (1,200 to 1,400 g). Moreover, these categories were adapted from the birth BW distribution of hyper prolific sows previously described (Quiniou et al., 2002; Škorput et al., 2018). During farrowing, when the piglets were born, their individual BW was examined and if they matched into the light or average BW category, they were selected to obtain samples. Selected piglets were removed from the sow before colostrum consumption and euthanized by an overdose of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Madrid, Spain). The entire liver was extracted and rinsed in phosphate-buffered saline solution to eliminate blood residues. Approximately 3 g of liver was collected in cryotubes and immediately frozen at −80 °C until antioxidant analysis. The remaining liver and left leg were collected and immediately frozen at −20 °C until mineral analysis. Samples of jejunum for gene expression analyses were taken only from neonate piglets of ITM and Replace sows. One sample of about 1.5 cm was collected, approximately at the midpoint of jejunum, and then rinsed in PBS solution, and later snap frozen in 1 mL of RNA (Deltalab, Barcelona, Spain), and stored at −80 °C for gene expression analysis.

Chemical analysis

Analytical determinations of diets were performed according to the AOAC International (2005) methods for dry matter (Method 934.01), Dumas method for crude protein (Method 968.06), traditional Soxhlet extraction method for ether extract (Method 920.39), and ash (Method 942.05). Neutral detergent fiber was analyzed using the Ankom nylon bag technique (Ankom 200 fiber Analyzer, Ankom Technology, Macedon, NY).

Mineral analysis

Mineral content was analyzed in feed, liver, bone, colostrum, and serum. Samples were processed as described in Villagómez-Estrada et al. (2021). Briefly, samples of feed were milled at 0.5 mm before mineral analysis. Liver was dried in a forced air oven at 102 °C for 12 h and then milled at 0.5 mm. The left leg was dissected, and tibia was autoclaved to remove all the adjacent muscle, tissue, and the fibula bone (121 °C for 30 min). The tibia bone was then oven-dried for 12 h at 102 °C and soaked in acetone under a chemical hood for 48 h to extract fat. After this period, the bone was again oven-dried for 12 h at 102 °C and then broken in the middle before being ashed in a muffle furnace overnight at 550 °C. Samples of colostrum were freeze dried at −55 °C and 0.06 mbar for 72 h (model Telstar, Fisher Scientific S.L., Madrid, Spain). Prior to mineral analysis, samples of feed, liver, colostrum, and bone ash were digested with concentrated nitric acid (65%) in a microwave oven (model Ultrawave, Milestone Srl, Sorisole, Italy). Serum samples were diluted in 1:20 volume ratio with 5 mL of a solution constituted by 0.05% ethylenediaminetetraacetic acid and 0.5% of ammonia. Colostrum samples were additionally mixed with deionized water before mineral analysis. All samples were analyzed by inductively coupled plasma-mass spectroscopy (model 7500 Agilent, Santa Clara, CA).

Antioxidant and oxidant analysis

For liver antioxidant analysis, approximately 1 g of liver was weighted and homogenized on ice with 4 mL of buffer solution using a Polytron homogenizer (model T18 digital Ultra-turrax, IKA, Staufen, Germany). The buffer solution used for homogenization consisted of 109.5 g/L of 0.32 M of saccharose (Merck, Darmstadt, Germany), 1.21 g/L of 10 mM Tris (Merck), and 0.37 g/L of the phosphate ethylenediaminetetraacetic acid buffer (Merck). Hydrochloric acid (37%; Merck) was used to adjust the solution at pH 7.4. The total homogenate was centrifuged at 4 °C at 1,500 *g* for 15 min to obtain a supernatant for the antioxidant enzyme analysis. The activity of glutathione peroxidase (**GPX**) and the malondialdehyde content in supernatant were determined by spectrometry, and following, respectively, the instructions of Ransel kit (Randox, County Antrim, UK) and Cayman kit (Chemical, Ann Arbor, MI). The GPX activity was referred to the total protein content in the supernatant and was determined by the Bradford (1976) method using the Bio-Rad protein assay reagent (Bio-Rad, Santa Clara, CA).

Gene expression analysis

For a complementary evaluation of the effect of maternal diet on fetal programming, a gene expression analysis of jejunum tissue was performed. The expression of 56 genes was studied by using a self-designed Open Array Real-Time PRC Platform (Applied Biosystems, Waltham, MA). Genes involved in multiple physiological functions closely related to intestinal health were selected based on the literature, and grouped based on their main function as follows: 1) barrier function genes such as the family members of claudins, mucins, and occludins (*OCLN*, *ZO1*, *CLDN1*, *CLDN4*, *CLDN15*, *MUC2*, *MUC13*, and *TFF3*); 2) immune response genes such as pattern recognition receptors, cytokines, chemokines, and stress proteins (*TLR2*, *TLR4*, *IL-1β*, *IL6*, *IL8*, *IL10*, *IL17A*, *IL22*, *IFNG*, *TNF*, *TGF-β1*, *CCL20*, *CXCL2*, *IFNGR1*, *HSPB1*, *HSPA4*, *REG3G*, *PPARGC1α*, *FAXDC2*, and *GBP1*); 3) antioxidant enzyme genes (*GPX2*, *SOD2*); 4) digestive enzyme and hormone genes involved in the digestion and metabolism process (*ALPI*, *SI*, *DAO1*, *HNMT*, *APN*, *IDO1*, *GCG*, *CCK*, *IGF1R*, and *PYY*); 5) nutrient transport coding genes (*SLC5A1*, *SLC16A1*, *SLC7A8*, *SLC15A1*, *SLC13A1*, *SLC11A2*, *MT1A*, *SLC30A1*, and *SLC39A4*); and 6) stress response genes (*CRHR1*, *NR3C1*, and *HSD11B1*); additionally 7) four reference genes were evaluated (*ACTB*, *B2M*, *GAPDH*, and *TBP*).

The RNA gene expression analysis for jejunum tissue was performed according to the methodology described by Reyes-Camacho et al. (2020). Briefly, RNA was obtained from 50 mg of frozen jejunum tissue using the RiboPure kit (Ambion, Austin, TX) and following the manufacturer's instruction. The quality and quantity of RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE), whereas the RNA integrity was checked with Agilent Bioanalyzer-2100 equipment (Agilent Technologies, Santa Clara, CA). Reverse transcription of approximately one microgram of total RNA to single-stranded cDNA in a final volume of 20 µL was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and random hexamer primers. The thermal cycler conditions applied were as follows: 25 °C 10 min; 37 °C 120 min; 85 °C 5 min; 4 °C hold. A total of 25 ng of cDNA sample was pre-amplified using a 2X TaqMan PreAmp Master Mix and a 0.2X Pooled Taqman Gene Expression Custom Assays in a final volume of 10 µL under the following thermal cycling conditions: 10 min at 95 °C; 14 cycles of 15 s at 95 °C, and 4 min at 60 °C, and a final step of 10 min at 99 °C. The pre-amplified cDNA product was stored until use at −20 °C. One replicate per sample was run in a Taqman Open Array gene expression custom plate format for gene expression, with 56 assays of 48 samples per plate (OpenArray plate) in a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems).

Gene expression data analysis

Data were collected and analyzed using the ThermoFisher Cloud software 1.0 (Applied Biosystems) applying the $2^{-\Delta\Delta Ct}$ method for relative quantification and using the sample with the lowest expression as a calibrator. The maximum cycle relative threshold allowed was adjusted at 26, amplification score < 1.240, quantification cycle confidence > 0.80, and the maximum SD allowed between duplicates was set at <0.38. Samples that did not meet these criteria or showed an inadequate amplification were removed. All data were subjected to a logarithmic transformation to get closer to the Gaussian distribution. Statistical analyses were performed using R software v.3.4.3 and Bioconductor software (Gentleman et al., 2004).

Statistical analysis

Data were analyzed with ANOVA by using the GLIMMIX procedure of SAS (version 9.4, SAS Institute, Cary, NC). The experimental unit for sow performance parameters was the individual sow. The model included the fixed effects of diet and parity group, as well as the random effect of gestation pen. Initial BW (day 35) was used as covariable for the analysis of BW at day 110. The standardized sow BW was calculated by subtracting the litter BW from the sow BW at day 110. Sow weight losses attributed to placenta tissue or fluids were not considered. Sow reproductive performance included total number of piglets born, born alive, stillborn, as well as the weight of alive litter and the average of alive piglet weight. Reproductive performance at farrowing was related to the sow. Colostrum and sow serum mineral content were analyzed with sow as experimental unit, and parity number as random effect. The experimental unit for mineral content and antioxidant enzymes of newborn tissues was the individual piglet nested within the sampled sow and was analyzed considering the sow diet, BW piglet category, and the interaction between sow diet × BW piglet category as main factors. The normality and homogeneity of the data were examined using the Shapiro–Wilk test and assessing the normal plot before statistical analysis. The normal adjustment was carried out for sow performance data, such as BW, back-fat, feed intake, tissue mineral content, and reproductive performance. Significantly different means were found using Tukey adjust. Significance was declared at a probability $P \leq 0.05$, and tendencies were considered when P-value was between >0.05 and <0.10.

Gene expression data were analyzed with ANOVA, using sow diet and BW piglet category as main factors. The Benjamini and Hochberg false discovery rate (**FDR**) multiple testing correction was also performed (Benjamini and Hochberg, 1995). The variability comparison between the experimental diet and the residual variability within diets is expressed as statistical contrast value. The differences between experimental diets were determined at P-values ≤ 0.10 and at FDR values ≤ 0.20. Heat map visualization was performed using the heatmap.2 function of the gplots package of R (Warnes et al., 2020).

Results

The analyzed TM content in the experimental diets is shown in Table 2. The expected differences in TM content between diets were achieved with all sources.

Reproductive sow performance

Sow productive and reproductive performance are shown in Table 3. The number of sows allotted per experimental diet at farrowing was 78, 78, and 77 for ITM, Replace and R&R, respectively. Difference with the initial number of sows corresponds to sows that showed physiological issues: lameness injuries, sudden dead, and abortion. Since the present study aimed to reflect results under commercial conditions, it was difficult to obtain an equal parity number between the experimental diets. Therefore, this factor was considered within the statistical analysis. No differences ($P > 0.10$) were observed between experimental diets on sow BW, back-fat thickness, or average daily feed intake. It is necessary to emphasize that the reported feed intake during gestation phase is the real consumption including the individual adjustment, which is carried out as a common commercial practice in the management on gestating sows. Reproductive performance was not affected by experimental diets (Table 3).

Mineral contents in sow and newborn piglet tissues and colostrum

Serum TM content of sows was not affected by experimental diets, except on Mn content (Table 4). Sows fed Replace and R&R diets had higher Mn content than sows fed ITM diet ($P < 0.001$). Colostrum Mn and Fe contents were higher in sows fed Replace diet compared to sows fed R&R ($P < 0.05$). Likewise, colostrum Cu content tended to be higher in sows fed Replace diet than that from sows receiving the other diets ($P = 0.080$). No differences in colostrum Zn content were observed ($P > 0.10$).

Piglets TM content was not affected by the two-way interaction between sow diet × BW piglet category ($P > 0.10$); therefore, only the main effects are shown in Table 5. Sow diet has no influence on TM content of piglet tissues, except

Table 2. Analyzed TM content in gestation experimental diets[1]

	Experimental diets		
TM element, mg/kg	ITM	Replace	R&R
Zn	130	148	113
Cu	30	30	26
Mn	90	99	88

[1] Feed samples, from each diet, were collected from four manufacturing batches and mixed into one representative sample. One feed sample from each diet was analyzed by duplicated. The total supplemented levels on ITM and Replace diets of Zn, Cu, and Mn with both mineral sources were 80, 15, and 60 mg/kg, respectively. The Zn, Cu, and Mn total supplemented levels on R&R diet were 64, 12, and 48 mg/kg, respectively.

for a tendency in tibia ash percentage. Piglets from sows fed ITM tended to have higher ash percentage than piglets from sow fed R&R diet ($P = 0.079$). The effect of BW category was evident on tissue TM content ($P < 0.05$). Light piglets had lower Mn serum content, and Zn, Cu, and Mn tibia content than average piglets ($P < 0.05$). Average piglets had lower tibia ash percentage compared to light piglets ($P = 0.029$).

Oxidant and antioxidant activity

Piglets born from sows fed Replace diet had a higher GPX liver activity than piglets from sows fed R&R diets, whereas pigs from ITM sows were intermediate ($P = 0.046$; Table 5). No interactions were observed between sow diet × BW piglet category on the activity on GPX or MAD content in liver ($P > 0.10$; Table 5). Likewise, no differences were observed in GPX activity (0.61 vs. 0.60 U/mg protein) and MAD content (30.9 vs. 30.2 µM) between light and average piglets ($P > 0.10$; Table 5).

Jejunum gene expression

A total of 46 genes were successfully amplified from either ITM or Replace diet. As BW at birth is critical for the piglet development, differences in mRNA gene expression were examined between light and average littermates of ITM and Replace sows (Table 6). The number of genes which show differences ($P < 0.10$) in mRNA gene expression between light and average littermates was lower in piglets born from Replace sows (five genes) than in those from ITM sows (nine genes). In general, light piglets showed a downregulation of genes involved in barrier, immune, and digestive functions compared with their average littermates.

The influence of maternal dietary intervention was shown by the expression of 19 genes in neonate piglets ($P < 0.10$; Figure 1). Piglets born from sows fed Replace diet had a higher jejunum mRNA expression of genes of Barrier function (*CLDN15*, *MUC2*, *MUC13*, *ZO1*), Immune response (*CXCL2*, *FAXDC2*, *HSPA4*, *HSPB1*, *IL-1β*, *IL8*, *REG3G*, *TGF-β1*), Antioxidant function (*GPX2*, *SOD2*),

Table 3. Reproductive performance of sows fed diets with total ITM, Replace, and R&R

	Experimental diets[1]				
Item	ITM	Replace	R&R	SEM	P-value
Sows, *n*	78	78	77	—	—
Parity, *n*	4.78	3.59	5.03	—	—
Sow BW, kg					
Gestation (day 35)	248.83	241.17	244.59	3.394	0.253
Gestation (day 110)[2]	266.99	263.27	266.20	1.631	0.231
Farrowing standardized[3]	246.30	242.58	244.80	1.625	0.247
Sow back-fat, mm					
Gestation (day 35)	14.54	14.53	14.41	0.423	0.968
Gestation (day 110)	10.41	9.96	9.83	0.222	0.121
Total feed intake, kg	177.13	178.68	176.3	0.923	0.192
Average daily feed intake, kg	2.33	2.35	2.32	0.012	0.191
Reproductive parameters					
Total born piglets, *n*[4]	19.07	19.14	19.27	0.472	0.951
Piglets born alive, *n*	16.17	16.38	15.98	0.416	0.790
Born alive litter weight, kg	20.97	20.83	21.43	0.526	0.703
Born alive average BW, kg	1.33	1.30	1.34	0.027	0.608

[1] ITM, inorganic trace mineral; Replace, partial replacement of trace mineral source; R&R, reduce and replace mineral source.
[2] Sow BW at day 35 used as covariate.
[3] Sow BW at day 110 excluding litter birth weight.
[4] Total born includes born alive and stillborn piglets.
[a,b] Means within a row with different superscripts indicate significant differences ($P < 0.05$).

Table 4. TM content of colostrum and serum of sows fed diets with total ITM, Replace, and R&R[1]

	Experimental diets[2]				
Item	ITM	Replace	R&R	SEM	P-value
Serum[3]					
Zn, mg/L	0.68	0.77	0.79	0.048	0.187
Cu, mg/L	1.64	1.68	1.58	0.043	0.283
Mn, mg/L	1.50[b]	2.41[a]	2.85[a]	0.185	<0.001
Fe, mg/L	1.29	1.35	1.49	0.122	0.462
Colostrum[4]					
Zn, mg/L	14.62	12.83	13.00	0.715	0.143
Cu, mg/L	4.40	4.54	3.85	0.238	0.080
Mn, µg/L	0.017[ab]	0.023[a]	0.016[b]	0.002	0.017
Fe, mg/L	1.41[ab]	1.63[a]	1.19[b]	0.071	<0.001

[1] Data are means of 20 sows sampled per experimental diet.
[2] ITM, inorganic trace mineral; Replace, partial replacement of trace mineral source; R&R, reduce and replace mineral source.
[3] Serum at day 110 of gestation.
[4] Basis on fresh matter.
[a,b] Means within a row with different superscripts differ significantly at $P < 0.05$.

Table 5. Tissue TM content and liver antioxidant activity of newborn piglets of sows fed diets with total ITM, Replace, and R&R[1]

	Experimental diets[2]				BW piglet category			P-value		
Item	ITM	Replace	R&R	SEM	Light	Average	SEM	Diet	Category	Diet × category
BW, g	1,049	1,043	1,012	34.366	721	1,348	24.996	0.714	<0.001	0.806
Liver, mg/kg DM[3]										
Zn	85.46	101.76	118.17	16.729	94.61	108.98	10.634	0.385	0.145	0.857
Cu	161.60	142.04	170.01	13.612	153.50	162.26	8.928	0.324	0.316	0.225
Mn	5.46	5.59	5.43	0.339	5.15	5.84	0.220	0.933	0.003	0.285
Fe	0.72	0.70	0.72	0.067	0.71	0.71	0.053	0.975	0.986	0.534
Tibia bone[4]										
Zn, mg/tibia	98.40	86.37	84.31	6.387	66.31	113.07	4.461	0.253	<0.001	0.898
Cu, mg/tibia	1.40	1.39	1.26	0.099	0.93	1.76	0.074	0.537	<0.001	0.536
Mn, mg/tibia	5.34	5.02	4.62	0.284	4.03	5.97	0.216	0.220	<0.001	0.955
Ash, %[5]	60.72	59.42	59.09	0.575	60.42	59.07	0.418	0.079	0.029	0.852
Liver antioxidant										
GPX, U/mg protein	0.59^{ab}	0.65^{a}	0.56^{b}	0.027	0.61	0.60	0.021	0.046	0.627	0.956
MDA, µM[6]	29.52	31.70	30.47	1.729	30.89	30.23	1.170	0.666	0.601	0.411

[1] Data are means of 10 newborn piglets for each BW piglet category and per sow experimental diet.
[2] ITM, inorganic trace mineral; Replace, partial replacement of trace mineral source; R&R, reduce and replace mineral source.
[3] DM, dry matter.
[4] Total TM expressed as total content of ash bone.
[5] Bone ash as percent of the weight of dried and defatted bone.
[6] MDA, malondialdehyde.
[a,b] Means within a row with different superscripts differ significantly at $P < 0.05$.

Digestive enzymes (*ALPI*, *ANPEP*), Digestive hormones (*CCK*, *IGF1R*), and Nutrient transport (*SLC16A1*) compared with piglets born from ITM sows ($P < 0.10$; Figure 1).

In order to explore the influence of sow diet on gene expression of each piglet BW category (light and average), the analysis of gene expression is presented separately. Light piglets born from ITM sows showed a lower mRNA expression of 15 different genes involved in different physiological functions such as Barrier function (*CLDN15*, *MUC2*, *TFF3*, *ZO1*), Immune response (*FAXDC2*, *HSPB1*, *PPARGC1α*, *TGF-β1*), Antioxidant function (*GPX2*, *SOD2*), Digestive enzymes (*ALPI*, *ANPEP*), Digestive hormones (*CCK*, *IGF1R*), and Nutrient transport (*SLC39A4*) compared with light piglets born from Replace sows ($P < 0.10$; Table 7).

Likewise, average piglets born from ITM sows showed a lower mRNA expression of genes involved in Barrier function (*MUC13*, *ZO1*), Immune response (*GBP1*, *HSPA4*, *IL-1β*, *IL8*, *IL6*, *TGF-β1*), and Stress enzyme (*HSD11B1*) compared with average piglets from Replace sows ($P < 0.10$; Table 8).

Discussion

Effects of feeding strategies on sow reproductive performance

Since, in the present study, TM diets were offered from day 35 postmating, when the embryonic implantation and consequently litter size had already been established, it was expected that maternal nutrition had effects on the development and survival (born alive) of piglets rather than in the total number of piglets born. Despite that the reproductive sow performance in the present study was not affected by experimental diets, a numerical decrease on live born piglets, or an increase in those stillborn, were observed on R&R sows. Coincidentally, these sows showed a moderate increase in parity number than Replace sows, which may have influenced this parameter besides receiving the reduced TM supplementation. However, the incidence of stillborn piglets should be considered as a multifactorial challenge with different noninfectious causes such as genetic lines, maternal, piglet, and environmental factors (Vanderhaeghe et al., 2013). Among these different noninfectious causes, the farrowing length, sow age, and proper nutrition may be of interest to the present study. An increase in litter size has been associated with a prolonged farrowing, which, in turn, is directly related to an increased risk of hypoxia in piglets (Vanderhaeghe et al., 2013). This prolonged farrowing may be attributed on the one hand to sow parity: as parity is increased, there is a greater risk of sow having stillborn piglets (Vanderhaeghe et al., 2013), presumably attributed to a loss on uterine muscle tone due to poor mineral reserves or oxytocin secretion (Langendijk and Plush, 2019). Although some studies did not confirm this relationship (van Dijk et al., 2005; Oliviero et al., 2009), others suggested that the duration of farrowing may have a greater impact on stillbirths than parity itself (Vanderhaeghe et al., 2013; Udomchanya et al., 2019). On the other hand, in terms of nutrition, for both young and old sows, a proper supply of macro and micro-minerals is considered important for the effective contractions of muscles surrounding the uterus, improving the transport of fetuses through the birth canal (NRC, 2012; Theil, 2015). Although TM requirements for sows are not well established, another possible reason for this decreased alive birth rate may be the low dosage of TM supplied in the R&R sow diet. In this sense, Vallet et al. (2014) showed that the dietary supplementation of Zn (700 mg/kg), at higher levels than those recommended, decreased stillbirth rate from late gestation (day 80), probably by decreasing birth intervals. Regarding the influence of TM source, early studies reported that a complete supplementation of OTM in sow diets increased the total number of piglets, as well as live born piglets at farrowing, compared with the ITM (Peters and Mahan, 2008; Caine et al., 2009). Whereas Holen et al. (2020) reported no differences in the number of stillborn, born alive, or total born piglets when diets were supplemented with three Zn levels (125, 365, and 595 mg/kg) by combining two Zn sources (sulfate and chelated). Similarly, strategies such as replacing 50% of ITM by OTM (Tsai et al., 2020) or reducing

Table 6. Relative gene expression differences between light and average BW newborn littermates of sows fed diets with total ITM and Replace[1]

Experimental diets[2]	Function	Response	Gene	Light	Average	Contrast statistic[3]	P-value	FDR
ITM	Barrier function	Trefoil factor 3	*TFF3*	0.93	1.33	1.712	0.207	0.650
	Immune response	Fatty acid hydrolase domain containing 2	*FAXDC2*	1.70	3.12	8.356	0.010	0.138
		Guanylate binding protein 1	*GBP1*	0.32	0.06	3.944	0.062	0.319
		Heat shock protein 1	*HSPB1*	1.27	1.81	1.338	0.002	0.083
		Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	*PPARGC1α*	2.87	4.79	7.799	0.012	0.138
		Interleukin 6	*IL6*	1.18	1.44	0.437	0.517	0.820
		Tumor necrosis factor alpha	*TNFα*	2.67	3.64	0.855	0.367	0.650
	Digestive enzyme	Aminopeptidase-N	*ANPEP*	0.61	0.72	4.528	0.047	0.311
		Indoleamine 2,3-dioxygenase	*IDO1*	1.40	4.72	4.973	0.040	0.310
		Sucrase-isomaltase	*SI*	7.27	55.70	9.834	0.006	0.131
	Nutrient transporter	Solute carrier family 39 (Zn transporter) member 4	*SLC39A4*	1.31	2.20	5.704	0.028	0.258
	Stress enzyme	Hydroxysteroid (11-beta) dehydrogenase 1	*HSD11B1*	2.88	1.92	3.970	0.062	0.319
Replace	Barrier function	Trefoil factor 3	*TFF3*	1.80	1.06	6.314	0.023	0.589
	Immune response	Fatty acid hydrolase domain containing 2	*FAXDC2*	3.18	5.58	2.395	0.139	0.589
		Guanylate binding protein 1	*GBP1*	0.19	0.51	0.491	0.493	0.839
		Heat shock protein 1	*HSPB1*	1.96	1.68	1.909	0.184	0.634
		Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	*PPARGC1α*	4.06	4.98	1.612	0.220	0.634
		Interleukin 6	*IL6*	1.07	2.64	3.625	0.073	0.589
		Tumor necrosis factor alpha	*TNFα*	3.08	5.59	3.413	0.081	0.589
	Digestive enzyme	Aminopeptidase-N	*ANPEP*	0.82	0.79	0.026	0.873	0.990
		Indoleamine 2,3-dioxygenase	*IDO1*	3.34	8.33	1.735	0.204	0.634
		Sucrase-isomaltase	*SI*	9.52	50.86	3.977	0.062	0.589
	Nutrient transporter	Solute carrier family 39 (Zn transporter) member 4	*SLC39A4*	2.18	2.40	0.255	0.619	0.919
	Stress enzyme	Hydroxysteroid (11-beta) dehydrogenase 1	*HSD11B1*	2.11	2.92	4.033	0.060	0.589

[1] Data are means of 10 newborn piglets for each BW piglet category and per sow experimental diet. Only significant gene expression differences are presented in both treatments ($P < 0.10$). Gene expression values are presented as ratios of cycle relative threshold value for each gene normalized to that of the reference sample.

[2] ITM, inorganic trace mineral; Replace, partial replacement of trace mineral source; R&R, reduce and replace mineral source.

[3] Contrast statistic expresses the variability comparison between the experimental diet and the residual variability within diets.

the total dose of ITM to 80% by a complete supplementation with OTM (Ma et al., 2020) did not show differences on litter size parameters compared with ITM.

As mentioned before, one of the major challenges associated with large litters are the greatest presence of low-birth-weight piglets and the variability within the litter. In the present study, no influence of TM strategies was observed on litter weight nor average live piglet weight. However, recent studies shown equivocal outcomes. Supplementing Zn at higher levels (365 mg/kg) than in the present study (64 to 80 mg/kg), during late gestation, appears to enhance piglet birth weight (Holen et al., 2020). The authors showed that a mixture of Zn sulfate with Zn chelate at 365 mg/kg resulted in heavier piglets (1.42 kg) and less incidence of low-birth-weight piglets (11.6%) compared with those sows fed a mixture of Zn sulfate and chelate at 125 (1.38 kg; 15.3 %) or 595 (1.40 kg; 15.1 %) mg/kg. On the contrary, Ma et al. (2020) supplementing sows, from breeding until 21 d postpartum, with a reduced OTM diet of Zn (72 mg/kg), Cu (12 mg/kg), Mn (20 mg/kg), and Fe (72 mg/kg) did not observe differences in the weight at birth of the pigs, except for a greater number of piglets with a birth weight greater than 1 kg (10.47 vs. 9.83) compared with sows fed a complete ITM diet (Zn: 90, Cu: 15; Mn: 25; Fe: 90 mg/kg). Likewise, Tsai et al. (2020) supplementing sows the entire gestation period shown no effect of Zn (120 mg/kg), Cu (30 mg/kg), and Mn (50 mg/kg) as ITM or partially replaced by OTM (50%) on litter birth weight nor the number of piglets weighing less than 0.91 kg. Therefore, further studies should be conducted to investigate the optimal levels or combinations of TM to enhance neonate weight and survival.

In the present study, an improvement of some Mn, Fe, and Cu levels in serum and colostrum were observed in OTM replacement sows. During the perinatal phase, the nutrition of piglets relies entirely on the nutrition of the sow through placental and colostrum transfer. In hyperprolific situations, this perinatal transfer of nutrients might not be optimal (Mahan et al., 2009; Matte and Audet, 2020), and according to Matte and Audet (2020), there is an active placental transfer of Zn but limited for other TM, such as Fe, Cu, and Se. In the present study, no differences were observed between maternal

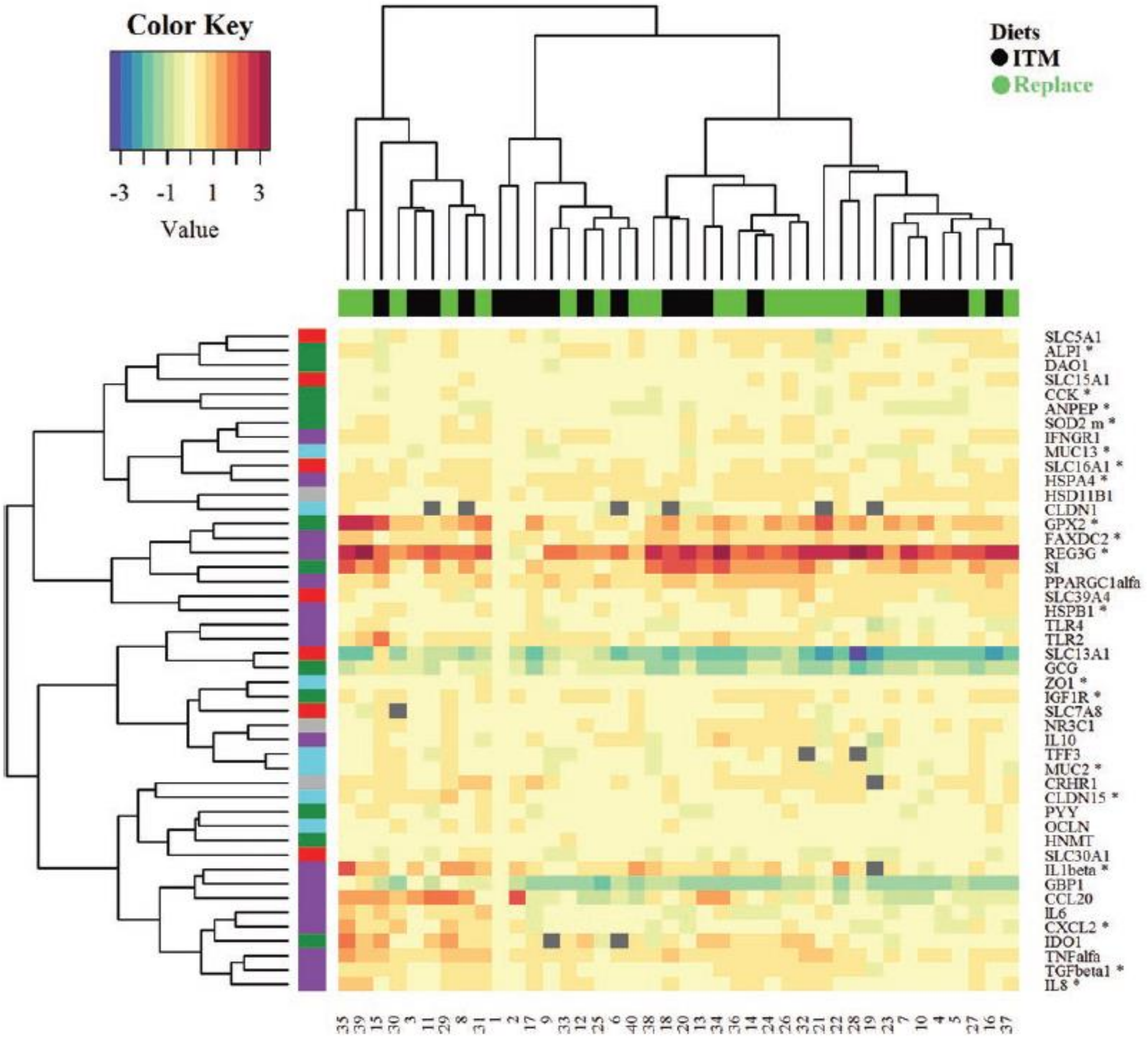

Figure 1. Heatmap and hierarchical clustering of gene expression levels of newborn piglets of sows fed diets with total ITM and partial replacement TM source (Replace). Significant differences are indicated using an asterisk symbol (P < 0.10). Data are means of 20 newborn piglets per sow experimental diet.

Table 7. Relative gene expression differences between light BW piglets of sows fed diets with total ITM and Replace[1]

Function	Response	Gene	ITM	Replace	Contrast statistic[2]	P-value	FDR
Barrier function	*Claudin-15*	*CLDN15*	1.36	2.37	6.312	0.022	0.143
	Mucin 2	*MUC2*	0.87	1.79	10.848	0.004	0.054
	Trefoil factor 3	*TFF3*	0.93	1.80	13.598	0.002	0.042
	Zonula occludens 1	*ZO1*	1.06	1.34	4.020	0.060	0.267
Immune response	*Fatty acid hydrolase domain containing 2*	*FAXDC2*	1.70	3.17	10.410	0.005	0.054
	Heat shock protein 1	*HSPB1*	1.27	1.96	18.122	0.0005	0.022
	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	*PPARGC1α*	2.87	4.06	3.179	0.091	0.301
	Transforming growth factor beta 1	*TGF-β1*	1.42	2.03	9.581	0.006	0.057
Antioxidant enzyme	*Glutathione peroxidase 2*	*GPX2*	9.08	22.64	3.034	0.099	0.302
	Superoxide dismutase	*SOD2*	1.17	1.56	7.290	0.015	0.112
Digestive enzyme	*Intestinal alkaline phosphatase*	*ALPI*	1.23	1.76	3.595	0.074	0.284
	Aminopeptidase-N	*ANPEP*	0.61	0.82	3.252	0.088	0.301
Digestive hormone	*Cholecystokinin*	*CCK*	0.85	1.35	4.076	0.059	0.267
	Insulin-like growth factor 1 receptor	*IGF1R*	1.53	2.22	4.756	0.043	0.246
Nutrient transporter	*Solute carrier family 39 (Zn transporter) member 4*	*SLC39A4*	1.31	2.18	3.896	0.064	0.267

[1] Data are means of 10 newborn piglets per each sow experimental diet. Only significant gene expression differences are presented (P < 0.10). Gene expression values are presented as ratios of cycle relative threshold value for each gene normalized to that of the reference sample.
[2] Contrast statistic expresses the variability comparison between the experimental diet and the residual variability within diets.

Table 8. Relative gene expression differences between average BW piglets of sows fed diets with total ITM and Replace[1]

Function	Response	Gene	ITM	Replace	Contrast statistic[2]	P-value	FDR
Barrier function	*Mucin 13*	*MUC13*	0.75	0.95	4.597	0.046	0.424
	Zonula occludens 1	*ZO1*	1.14	1.39	3.368	0.083	0.424
Immune response	*Guanylate binding protein 1*	*GBP1*	0.06	0.51	4.567	0.047	0.424
	Heat shock protein 4	*HSPA4*	2.02	2.70	4.475	0.049	0.424
	Interleukin 1 beta	*IL-1β*	2.39	18.23	4.140	0.058	0.424
	Interleukin 8	*IL8*	1.74	3.59	3.463	0.079	0.424
	Interleukin 6	*IL6*	1.43	2.64	3.406	0.082	0.424
	Transforming growth factor beta 1	*TGF-β1*	1.58	2.43	4.554	0.047	0.424
Stress enzyme	*Hydroxysteroid (11-beta) dehydrogenase 1*	*HSD11B1*	1.92	2.92	6.058	0.024	0.424

[1]Data are means of 10 newborn piglets per sow experimental diet. Only significant gene expression differences are presented ($P < 0.10$). Gene expression values are presented as ratios of cycle relative threshold value for each gene normalized to that of the reference sample.
[2]Contrast statistic expresses the variability comparison between the experimental diet and the residual variability within diets.

diets in neonatal piglet TM content. In the same way, Peters et al. (2010) indicated that the transfer of minerals to the fetus *in utero* was not greatly affected by the dietary source (ITM and OTM), or level of TM provided to sow. On the other hand, the higher mineral content in colostrum and serum from OTM replacement sows may suggest an improved postnatal transfer of nutrients for neonatal development compared to those fed the R&R diet. Similarly, Peters et al. (2010) reported that the OTM supplementation in sow diets resulted in the increase of Cu, Se, P, and Mg colostrum content compared with the ITM diets. Interestingly, although the dietary Fe source and level were kept constant in the three experimental diets, the colostrum Fe level was greater in replacement sows. Although, each TM has a specific pathway of intestinal absorption, a competition for *DMT1* transporter may occur between Fe, Zn, Cu, and Mn (Goff, 2018). Our finding may suggest a greater competition for transporters between ITM than with their chelated counterparts.

Since the present study was focused only on gestating sows, the performance of the sow and litter during lactation phase was not recorded. Further complementary studies that also consider the lactation phase could provide more information on the midterm effects of these dietary strategies on sow and piglet performance.

Differences between light and average BW piglets

It was observed that the tissue mineral content and the jejunum gene expression differed according to the BW of the neonate piglets, with light piglets being unable to store TM and express genes as effectively as their heavier littermates. According to Mahan et al. (2009), as litter size increases, large quantities of minerals would be expected to be transferred from the sow to litter, but this transfer may differ according to the neonatal BW. Indeed, it is widely accepted that intrauterine embryo crowding in large litters implies that the first embryos to implant can limit the physiological development of the embryos that adhere later, with this embryonic competition dramatically increasing with each successful embryo attachment (van der Waaij et al., 2010). The impaired physiological development of light piglets was also observed through the deficient expression of several genes implicated in metabolic and immune responses. A compromised gut can not only reduce the absorption of nutrients, but it also impairs the mucosal immune system, which is necessary to defend and restore the body's homeostasis (Weström et al., 2020). In fact, among the frequent complications of low-birth-weight piglets are, among others, the poor development of several organs and reduction in the wall thickness of gastrointestinal tract (Edwards et al., 2019). In humans, intrauterine growth restricted placentas have showed a reduced content of some TM such as Cu (23%) and Zn (37%) compared to normal neonate placentas (Zadrozna et al., 2009). Likewise, in pigs, the intrauterine growth restricted placentas have shown a greater oxidative damage and impaired angiogenesis compared with those heavier littermates (Hu et al., 2020). Overall, the outcome from the biomarkers evaluated in this study emphasizes the important maternal–fetal relationship, and in which the imbalance induces digestive and immunological disorders, particularly in light birth weight neonates.

Effects of maternal sow diet on piglet gene expression

Maternal nutrition not only plays not only a critical role in fetal growth and development but also is the major factor that alters expression of the fetal genome and hence may have lifelong consequences (Wu et al., 2004). The partial substitution of ITM by OTM in sow diets resulted in an upregulation of 15 genes implicated in intestinal barrier, immune, and antioxidant responses, particularly in light newborn piglets. Since Zn, Cu, and Mn are essential micronutrients involved in all major metabolic pathways (Suttle, 2010), it is possible that an improved maternal nutrition may influence the function of these TM on the progeny. Previous studies feeding sows with several types of nutrients (De Vos et al., 2014), as well as with different levels and sources of TM (Holen et al., 2020; Ma et al., 2020), have described positive effects on the birth weight of the piglets. However, none have described the positive effects that these maternal dietary interventions have on the fetal imprinting on the smallest piglets of the litter. The influence of maternal dietary intervention on gut health genes will be discussed in the following sections, according to their main physiological function.

An upregulation of genes involved in barrier functions (*MUC2*, *MUC13*, *ZO1*, *TFF3*, *CLDN15*) was observed in light and average neonate piglets born from sows fed Replace diet. An adequate intestinal barrier function involves multiple intestinal mechanisms, to ensure the defense against pathogenic microorganisms, such as immune cells, antimicrobial peptides, tight junctions, and mucins (Wijtten et al., 2011). Mucins and linker proteins like ZO-1, in particular, play an important role in the protective barrier within the gastrointestinal tract, safeguarding the intestine and its structural integrity (Zhang and Guo, 2009; Liu et al., 2014; Pelaseyed and Hansson, 2020). Among all TM, perhaps Zn is the most widely investigated with consistent evidence of its vital role in the regulation of intestinal integrity and immune function in live beings (Liu et al., 2014;

Chasapis et al., 2020). For instance, in female broiler breeders, the Zn supplementation in chelated form increased the *MUC2* expression in progeny (Li et al., 2015).

In general, as the intestinal mucosa possesses a complex function, it does not only play a role in nutrient absorption and epithelial barrier but is also part of a well-organized immune system (i.e., gut-associated lymphoid tissue; Ramiro-Puig et al., 2008; Weström et al., 2020). *GBP1* gene is involved in the control of inflammation and host immune response (Honkala et al., 2020), particularly against several bacterial, parasite (Selleck et al., 2013; Fisch et al., 2019), and viral pathogens (Khatun et al., 2020). Since the intestinal *GBP1* gene expression levels were similar in light piglets from both ITM and OTM diets, it seems that *GPB1* gene expression is especially required under critical circumstances. Moreover, when comparing the expression of *GBP1* gene between light and average littermates under ITM conditions it was also higher in light piglets. Whereas OTM diet seems to be able to induce an upregulation of this defensive molecule even in those average piglets, suggesting a strengthening immune effect until later in life. This immunoregulatory effect by the OTM diet is also observed for *TGF-β1* gene expression, a key mediator molecule in the mucosal immune system (Konkel and Chen, 2011), which was upregulated in both light and average piglets compared with those in the ITM group.

Moreover, the partial feeding of OTM in sows not only resulted in an upregulation of the above anti-inflammatory genes (*GBP1*, *TGF-β1*), but also of the proinflammatory ones (*IL-1β*, *IL-6*) in average piglets. A balance between the expression and function of anti-inflammatory and proinflammatory cytokines is necessary to rapidly detect invaders (proinflammatory) and to initiate an appropriate immune response (balanced proinflammatory and anti-inflammatory responses; Cicchese et al., 2018). For instance, in weaned piglets, the pharmacological doses of Zn downregulated the proinflammatory cytokine *IL-1β* and *IFN-γ*, while increasing the expression of *TGF-β* (Zhu et al., 2017).

Although the upregulation of anti-inflammatory and proinflammatory genes was observed in average piglets of both diets compared with their light littermates, the average OTM piglets seems to upregulate more anti-inflammatory genes than proinflammatory genes compared with the ITM piglets. Complementary studies focused on the specific influence of the dose and chemical form of TM in the stimulation or suppression of the expression of immune cells could help to decide the precise dose and form of TM to strengthen the immune system.

Interestingly, changes in the genes encoding antioxidant enzymes were observed only in light piglets, being upregulated in those born from Replace sows. Three key enzymes (superoxide dismutase, catalase, and GPX) constitute the first line of defense in neutralizing any free radicals (Ighodaro and Akinloye, 2018). TM such Zn, Cu, and Mn are extensively recognized as cofactors and constituents of these antioxidant enzymes (Suttle, 2010). In the present study, the increased activity of the GPX enzyme in the liver, along with upregulation of *GPX2* and *SOD2* mRNA levels in the intestine, may suggest an improved antioxidant capacity in piglets born from sows fed OTM, particularly in light piglets, compared with those born from ITM sows.

In the same way, changes in mRNA levels of digestive response genes were only detected in light newborn piglets. An upregulation of several digestive response genes (*ALPI*, *ANPEP*, *CCK*, *IGF1R*, *SLC39A4*) were observed on light piglets born from Replace sows, compared with those from ITM sows. In particular, *IGF-1* is known as the main mediator of the effects of the growth hormone, but also for its effects on the improved absorption of nutrients and electrolytes, and stimulation of the recovery of the intestinal epithelium (Li et al., 2016). Whereas the *SLC39A4* (*ZIP4*) gene is considered as the crucial factor for Zn dietary uptake from the gut lumen and in the control of the systemic Zn homeostasis (Lichten and Cousins, 2009; Hashimoto et al., 2016). The increased expression of this Zn transporter gene in light piglets born from the Replace OTM may be in line with the highest mRNA levels of all the genes mentioned above that need Zn for their expression.

In the light of the present results, it is concluded that the partial substitution of inorganic TM by their organic counterparts represents an alternative to the totally inorganic supplementation, with positive effects on neonate piglet gene expression. Although the lower TM storage, together with the greater downregulation of physiological genes, exposes the immaturity and vulnerability of the smallest neonates of the litter to cope with the challenges after birth, the partial supplementation of maternal diets with OTM may denote a nutritional strategy to mitigate the negative effects of the intrauterine fetal growth competition.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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CHAPTER VII

GENERAL DISCUSSION

Traditionally, the poultry (133.3 million tons) and pork (109.2 million tons) industries have led world meat production (FAO, 2021), mainly as strategies to reduce malnutrition and undernourishment of human populations due to their relatively low costs. However, livestock production has had to expand in order to meet the nutritional needs of the world's population, which has indirectly implied greater use of land (crops, farms) and water for animal husbandry but also an increase in the risk of environmental pollution. The swine industry currently faces two main challenges, namely raising animals under the restrictions in the use of antibiotics and pharmacological doses of mineral additives in feed (e.g. 2500 mg/kg Zn as ZnO), and pressure to reduce its environmental impact (e.g., slurry and gas emissions), not to mention the constant need to minimize production costs. In order to control the collateral consequences of swine production, the EU has proposed such legislative actions as: a) limitations on doses of Zn and Cu in feed, b) banning the pharmacological use of ZnO, and more recently c) devising the "green deal" project that pursues healthier and sustainable animal food without negatively impacting the environment (air, soil, water) and reducing the risk of life-threatening diseases. Therefore, today more than ever, the swine industry needs to be competitive and sustainable. Although this is not easy to achieve, animal nutrition might be the cornerstone for doing so. Indeed, precision feeding is one of the most promising methods to reduce excessive excretion of nutrients into the environment (manure) while respecting animal welfare and supporting growth performance rates, which not only complies with legislation but is also part of the social responsibility required of animal production.

In this regard, the present thesis assessed three alternative approaches to TM supplementation using different sources and with reduced doses in feed for the post weaning, growing and reproductive phases of pigs, with no other antimicrobial additives, while elucidating some of their associated mechanisms of action. All the studies were carried out on commercial farms, including the formulation of commercial feed (i.e. ingredients and enzymes), in order to understand these results under practical rather than strictly experimental conditions, for the latter may not reflect all the real challenges of the swine industry.

In essence, it was observed that reducing dietary levels of Zn and Cu, mostly through hydroxychloride source, decreased total fecal excretion and probably the pressure on bacteria to develop antimicrobial resistance mechanisms, while stimulating feed consumption to achieve acceptable growth rates, and also respecting animal health and welfare. In addition, maternal nutrition with a partial supplement of organic minerals improved fetal programming with possible long-term positive effects on the progeny. Thus, the aim of this general discussion is to highlight the most interesting findings in consideration the main initial hypothesis raised by this thesis.

Effects of reducing the supplementation levels of Zn and Cu, as a strategy to diminish the risks of environmental contamination, on pig growth performance

A reduction in the supplementation levels of Zn and Cu is undoubtedly a need for livestock sustainability. Nonetheless, achievement of acceptable performance rates at current reduced legislative levels, or even in the face of possible future reductions, is only possible via supplementation from highly available sources. From the animal's point of view, the most important parameter regarding the sources and doses of TM is the availability of the element that will be used first in normal physiological functions (growth, health, and reproduction) and later to enhance growth performance and thus achieve full genetic potential. As a result of the first experiment (**Chapter III**), a clear impact of Cu level (15 vs 160 mg/kg), with either a sulfate or hydroxychloride source, was denoted in the growth performance of newly weaned pigs (16.6 vs 17.7 kg BW), under environmentally challenging conditions. Although the reduction of Cu and Zn levels in the diet can have a significant impact on reduction of the final excretion of TM in the manure (as observed in **Chapter V**), the growth development of these stressed and immature weaned pigs was impaired when Cu levels in the feed were extremely low, close to those considered to be nutritional requirements, even in the presence of phytase enzyme. But even more importantly than the deterioration of commercial growth rates, these low nutritional levels could threaten pig physiology and therefore animal welfare. Weaning itself decreases plasma Cu and Zn levels, at least during the first two weeks after weaning (Carlson et al., 2007; Davin et al., 2013), which added to the low feed intake by pigs after weaning may not fully satisfy their normal metabolic and physiological requirements, being further injured if the food supplies a very low amount of these minerals. *The impact of this low level of Cu on the health and welfare* *of pigs after weaning must be seriously considered when proposing a further and more severe reduction in these minerals feed levels.*

In contrast, the reduction of the TM dose in growing pigs did not present the same picture as with weaned pigs, although the target mineral in this experiment was Zn (20 vs 80 mg/kg) with a lower participation of Cu (15 mg/kg) and with even lower feed TM levels than those established by EU regulations. In fact, the results indicated that the growth performance and carcass characteristics of grower-finisher pigs respond in a similar manner to both reduced Zn levels (0.415 vs 0.421 G:F), without clinical signs of Zn deficiency such as parakeratosis or decreased growth. The most plausible explanation for these results is the highly efficient homeostasis system of living beings. In general, when minerals are supplied above human or animal physiological needs, absorption efficiency decreases and endogenous losses even increase in order to achieve homeostatic balance (Goff, 2018; Ohashi et al., 2019). An excellent example is Zn regulation, which is addressed in more detail in Ohashi et al. (2019). As deficient or excessive Zn can critically disrupt physiological and metabolic cellular processes, its level and distribution must be highly regulated, mainly by transporter proteins that are responsible for controlling Zn influx (ZIP) and efflux (ZnT), which help to maintain cellular homeostasis (Ohashi et al., 2019). At this age, the requirement of TM to maintain homeostasis by pigs is likely to be relatively low, considering the lower physiological challenges (e.g. diet, pen mates, dysbiosis) as compared to those observed in the weaning phase. Moreover, at this stage, as well as having a mature immune system and a stable intestinal microbiome, pigs have greater feed intake; hence their mineral needs can be met as more feed is consumed. Additionally, although not completely understood, the effect of digestive enzymes such as phytase and carbohydrase in delivering the innate TM content of feed ingredients cannot be discarded.

To take this reduction concept a step further, recall that two of the current challenges for swine production are the excretion of TM into the environment and the development of bacterial co-resistance to antimicrobials. Our trials showed that reducing doses of Zn and Cu in feed decreases its total fecal content by 45.5% and 18.5% respectively (**Chapter V**), hence minimizing its pollution risks. Along similar lines, it was hypothesized that reducing TM in the feed could diminish the excessive antimicrobial pressure on bacteria communities to develop antimicrobial resistance mechanisms. However, high or low TM levels in weanling diets did not influence the presence of *tcrB* and *van* genes (*VanA*, *VanB*, *VanC1* and *VanC2*) while a low prevalence of *ermB* and *tetM* genes was recorded among 40 isolates of *Enterococcus*. Study of this potentially positive effect of reducing TM levels might require more extensive monitoring on different commercial farms of diverse sanitary status. On farms with poor sanitary conditions and an intensive history of antibiotic use, prolonged periods of antimicrobial withdrawal may be required in order to generate changes in the rates of antimicrobial resistance.

Just as important as the dose of TM is how it is supplied in diets. As mentioned earlier, in commercial practice there are different TM sources, which vary in price, characteristics, mineral content, and most of all bioavailability. The factors that might affect TM bioavailability in a living being include the concentration of the element, chemical form, nutrition and health of the individual, excretory losses, and nutrient-nutrient interactions (e.g., TM interactions, phytate complexes, among others) (Hambidge, 2010). Of these factors, the intrinsic relationship between the chemical form (solubility characteristics) and the interaction with other nutrients in the diet (particularly phytate) will dictate the amount of element that will certainly be available on the apical surface of the enterocytes to be absorbed. Phytate (phytic acid; inositol hexa- (or penta)-phosphate) is widely known in both human and animal nutrition as an outstanding dietary factor that impairs the bioavailability of the ingested TM, especially for Zn; whose dietary requirement in humans (6 mg/day) can double in the presence of 1 gr of phytate (Hambidge, 2010). With this in mind, two *in vitro* assays were developed (**Chapter IV** and **V**) in similar conditions to those found in the GIT, including the presence of phytic acid. As a result, for instance, Cu solubility from a hydroxychloride source decreased radically from 100% at pH 2.5 and 4.5 to 9% at pH 6.5 (intestinal pH), while Cu sulfate solubility persisted at around 98% in the whole range of pH levels (2.4-6.5). When phytic acid was added, Cu solubility decreased as pH increased (from 99% at pH 2.5 to 21% at pH 6.5), being more critical with Cu sulfate at intestinal pH, thus corroborating the susceptibility of sulfate to be bound by phytate and probably by other minerals. The absorption of TM takes place in the small intestine, particularly in the duodenum and jejunum, and depends on the degree of mineral dissociation from the diet (Matte et al., 2017; Ohashi et al., 2019). It is, therefore, likely that the efficiency of intestinal absorption of hydroxychloride minerals is superior to the sulfate counterparts. Nonetheless, when weaned and growing pigs were supplemented with sulfate or hydroxychloride minerals, both sources were equally efficient in supporting growth. However, when evaluating animal physiology through sensitive biomarkers such as antioxidant enzyme activity, mineral content in serum, liver, and bone tissues and lately in the modulation of colon microbial communities, a positive regulation was recorded from the hydroxychloride source, particularly with the Cu element (Figure 3). Additionally, this source effect was observed in the carcass characteristics of fattening pigs, where hydroxychloride minerals promoted a greater carcass yield and a higher apparent total digestibility of Zn and Cu when Zn was supplemented at 80 mg/kg. Although the retention rates were not measured, this greater apparent digestibility, together with other subclinical improvements, may suggest greater intestinal absorption, which may instead contribute to general health and carcass improvement. Indeed, previous studies on pigs and broilers have reported an increased bioavailability of Zn and Cu as hydroxychloride compared to sulfate counterparts (111-112%) and a more prominent bioavailability than Zn oxide (159.3%) (Miles et al., 1998; Batal et al., 2001; Mavromichalis et al., 2001; Luo et al., 2005; Zhang and Guo, 2007).

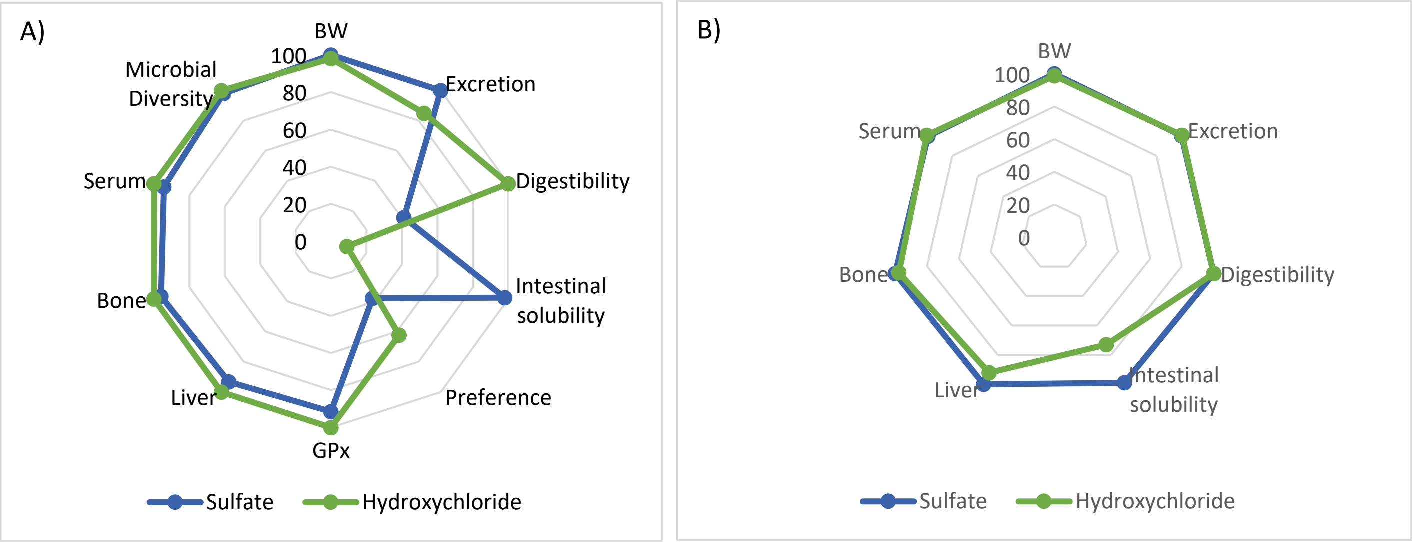

Figure 3. Comparison between sulfate and hydroxychloride Cu (A) and Zn (B) source according to the multiple response variables evaluated in Chapter III to V.

Practically, these observations support the proposal that feeding reasonably low Cu and Zn levels to pigs, mostly as a hydroxychloride source, is a valuable strategy to support growth performance and health while reducing fecal mineral excretion and controlling the increase in antimicrobial resistance rates that could threaten public health.

Moderately high levels of Cu in post-weaning diets impact on pig feed intake stimulation and intestinal microbial modulation

At the time of weaning, the pig's GIT is rapidly evolving with significant changes in gut structure, microbial colonization, digestion function, and immune development (Choudhury et al., 2021). Indeed, the role of GIT is more complex than it seems, for in addition to being the largest immune organ in the body, its purpose is to detect whether ingested feed contains nutrients, as well as potential non-nutrients, such as toxins. This ability to mediate responses according to dietary content has been shown to have a tremendous impact on the hunger-satiety cycle, since there is an intrinsic relationship between the nutrient sensors and/or transceptors located along GIT (e.g. taste and chemosensory receptors) and the enteroendocrine system (e.g. CCK or PYY) (Roura et al., 2019). Therefore, on route to achieving target growth rates by stimulating feed consumption, the main focus must be the intestinal system and its nourishment, especially in young pigs. In this sense, Cu could be a reliable strategy to stimulate feed intake in critical periods, such as weaning, mainly due to its systemic effects related to fasting neuropeptides, as reviewed at the beginning of this dissertation. Nevertheless, based on human studies, it is known that Cu has a bitter or metallic taste, which can potentially result in aversion to food intake because the GIT associates the bitter sensation with harmful or toxic compounds, thus initiating defensive responses such as vomiting, inhibition of gastric motility, and an increased feeling of satiety (Roura et al., 2019). In commercial practice, pigs are not given the opportunity to select their feed, so it cannot be determined whether these systemic signals, sent by nutrients, can exert changes in animal feeding behavior. Since one of the primary objectives of veterinarians and swine producers is to stimulate immediate feed consumption after weaning, this experiment was designed to assess whether a supplemented Cu source (sulfate vs hydroxychloride) or level (15 vs 150 mg/kg) could produce aversion to feed because of the taste of Cu or whether, on the contrary, a preference is triggered due to its systemic effect. When comparing Cu sources, it was observed that pigs immediately preferred feed supplemented with Cu as hydroxychloride than in sulfate form (62.2 vs. 37.8%; **Chapter IV**). Given that pigs have a high sensory capacity, and that observed differences in solubility (*in vitro* experiment) between TM sources can affect their taste (human studies; Zacarias et al., 2001; Cuppett et al., 2006), it is plausible to consider that the Cu hydroxychloride source tastes less bitter than Cu sulfate, which may have reduced feed rejection by weaned pigs. However, and as mentioned earlier, an animal's preference for a particular feed or nutrient involves a complex enteroendocrine system that integrates the experience of taste with its post-ingestive effects. Thus, surprisingly, the feed with moderate-high levels of Cu (150 mg/kg) stimulated greater feed consumption by the piglets than the low-nutritional dose of Cu (15 mg/kg). The most reasonable explanation for this preference is the physiological need to re-establish Cu homeostasis, which is fragmented after weaning, orchestrated by positive feedback driven by the neurological signaling pathways of feeding (e.g. NPY; Li et al., 2008; Zhu et al., 2011), thus resulting in a systemic effect on feed intake. In nutrient deficit scenarios, pigs have already been able to modulate their food preference (Forbes, 2010) which, added to the results presented by this thesis, reinforces the high efficiency of their specialized neuroendocrine system. *The practical value of this greater preference for feed could be related to earlier recovery of the physiological void left by weaning in intestinal development and normal physiology, in addition to minimizing anorexia, dehydration and dysbiosis, hence providing a greater state of well-being to the pig. Therefore,* *inclusion of this reasonably high Cu dose in post-weaning feed could be justified over only supplementing with the low nutritional dose.*

At the same time that the structure and function of the weaned pigs' GIT is maturing, so is the intestinal microbial population and diet may play a central role in this. For many years, one of the hypothetical mechanisms of action attributed to pharmacological doses of Cu, particularly in weaned pigs, it has been its antimicrobial effect. In recent years, novel biotechnological tools that use high-throughput elemental profiling technologies (e.g., metagenomic sequence, transcriptomic, proteomic, etc.) have led to a better understanding of the biological mechanisms of different nutrients on the host, such as Cu action on microbial communities. Thus, using metagenomic sequence (16-S) it was possible to further understand the effect of high doses of Cu in diversity (i.e., abundance) and evenness (i.e., balance) of microbial communities (**Chapter III**). The results indicated that Cu supplementation at 160 mg/kg reduced the diversity (Shannon and Inverse Simpson indexes) of the colonic microbial community at a clear distance from the nutritional (15 mg/kg) dose group. Whether lower microbiota diversity is considered beneficial or not for growth goals remains uncertain; however, as long as the swine gut maintains a healthy microbiome, preventing pathogens and promoting beneficial bacteria abundances, it will be beneficial to animal growth. Indeed, an in-depth analysis of microbiota communities showed that the relative abundance of some genera such as *Roseburia* increases while *Streptococcus* and *Lactobacillus* decrease with high Cu addition. Interestingly, the Cu source seems to affect microbial populations differently, since the abundance of genera such as *Lachnospira* and *Roseburia* increased, while *Streptococcus* and *Escherichia* decreased with a high dose of Cu as hydroxychloride compared to Cu sulfate at the same level. Considering the antimicrobial mechanisms proposed for Cu and Zn, this bacteria profile was not surprising, although it must be noted that this antimicrobial effect seems to be stronger for some bacterial groups, which indirectly favors the growth of other bacteria that, judging by these results, are mainly saprophytes. The microbial community that inhabits the GIT has a symbiotic relationship with the host, which provides a comfortable environmental niche but in return receives benefits such as the fermentation of nondigestible starch into SCFA, such as butyrate. In particular, butyrate is a preferred energy source for colonic enterocytes and thus potentially contributes to intestinal homeostasis (Ríos-Covián et al., 2016). Some of the family and genera members (e.g. *Roseburia*) that increased as a result of high Cu supplementation are known to directly or indirectly produce SCFA. It is therefore reasonable to contemplate that this bacterial modulation, aimed at the reduction of opportunistic pathogens together with the development of saprophytic bacteria, could lead to a significant improvement in intestinal nutrient absorption and, therefore, pig feed efficiency as observed in this dissertation.

With these points in mind, feeding pigs reasonably high levels of Cu and Zn, particularly in critical phases such as post-weaning, is a useful approach not only to improve performance and health, but also to reduce the period of anorexia by stimulating feed consumption.

Feeding gestating sows with functional doses and sources of trace minerals might have a positive impact on neonatal development and programming

Taken together, the maternal genetic and epigenetic effects show the successful trajectory of the progeny and as such the nutritional impact of sows on the progeny is invaluable, especially in modern hyperprolific situations. In the last experiment developed in this thesis (**Chapter VI**), a partial replacement of inorganic minerals with organic counterparts, together with a reduction of the TM dose, was assessed to minimize feed costs and reduce fecal mineral excretion without compromising TM bioavailability for animal and offspring. Three approaches were established, namely a) complete dose of inorganic trace minerals of Zn, Cu and Mn; b) replacement of inorganic trace minerals with organic counterparts at 30% of the complete dose; and c) a reduction by 50% of the inorganic trace mineral dose. Although the reproductive performance of gestating hyperprolific sows was not affected by experimental diets, sows fed the partial replacement diet showed an improved TM content for serum and colostrum, hence suggesting an enhanced absorption of TM that may be available for neonate pigs after farrowing. When the progeny was evaluated, no differences in tissue (liver and bone) TM storage were detected, although evaluation of other sensitive biomarkers, such as antioxidant enzyme activity and the expression of functional genes, evidenced the influence of maternal nutrition. Partial supplementation with organic minerals supported the neonatal expression of several functional genes involved in immune response, barrier and antioxidant function, and digestive processes compared to those born from sows fed the totally inorganic diet, with particular emphasis on the smallest pigs in the litter. Some examples of this improved gene expression are shown in Figure 4, while the more detailed results are presented in [Chapter VI](#).

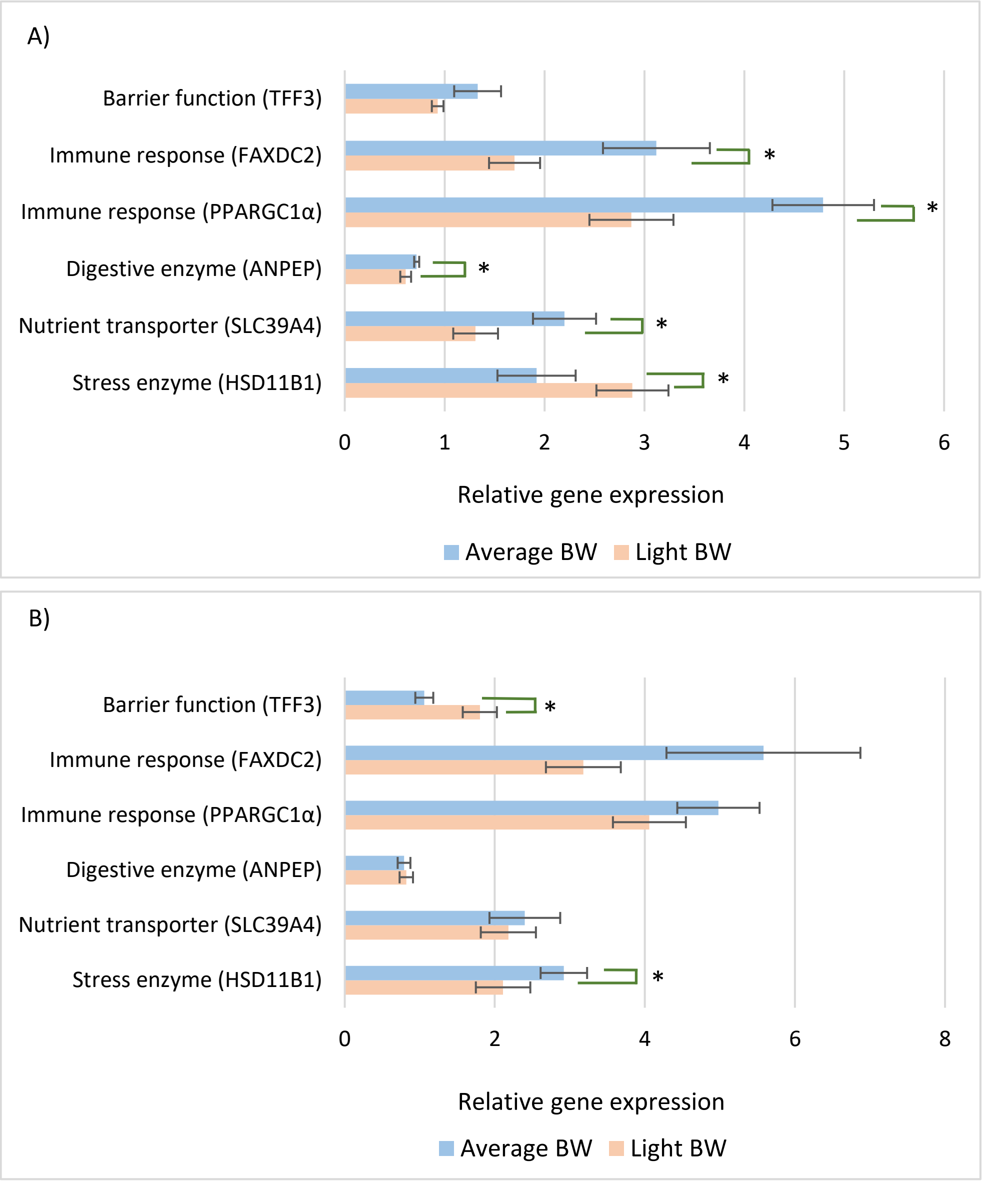

Figure 4. Relative gene expression of neonate pigs born from sows fed diets with total inorganic trace mineral (A) and partial replacement trace mineral source (B). Asterisk symbol (*) indicates significant gene expression differences ($P < 0.10$).

To further understand the value of these results, recall that perhaps the most critical disadvantage of large litters is the reduction and variability in birth weights of neonate pigs, and this alone has been demonstrated to have a major impact on pig survival during the suckling period and in later performance. With this in mind, in our experiment the neonates were classified into two BW categories (light and average) to be sampled in order to evaluate the influence of the BW condition but also the influence of maternal nutrition on their fetal programming. As expected, small or light pigs exhibited larger immaturity and vulnerability compared to their heavier littermates, with poorer expression of genes responsible for regulating vital functions such as immunity, digestion, and intestinal barrier function. Nonetheless, supplementing mothers with the blend of inorganic and organic minerals partially alleviated these downregulations compared with a completely inorganic dose (e.g. *ZO1* mRNA levels (1.34 vs 1.06)). These findings can be interpreted as aiding these small newborns to cope with postnatal challenges. To our knowledge, this is the first study to evaluate this TM approach to fetal programming, with particular interest regarding the smallest neonates.

For a better understanding of the impact of these genomic results on future pig performance, as a consequence of maternal diets, note that fetal programming is the genomic process that occurs during embryonic and fetal development, alteration to which causes different readings by cells when it comes to producing the required amino acids to build proteins (Kwon and Kim, 2017). Thus, in mammals, and particularly in pigs, fetal nutrition and programming could be conditioned by several maternal factors such as nutrition, genetics, parity or age, body composition and utero-placental blood flow. In critical circumstances, when maternal nutrients are not completely satisfied, an undernourished fetus will not only result in lower weights at birth but also in long-term effects such as adverse impacts on organ structure and function (e.g. brain, intestine), delayed growth, reduced feed efficiency, and impaired meat quality, as has been classically observed in intrauterine growth restricted pigs (Wang et al., 2017). In such circumstances, the present observations support the importance of supplying adequate micronutrients, such as Zn, Cu and Mn, in maternal nutrition in order to support not only fetal development, but also long-term fetal imprinting related to functional activities such as immunity and digestion. Although not assessed in this study, quantifying the levels of minerals excreted by these pregnant sows would have provided more information on the benefits of these mineral supplementation strategies in reducing the environmental impact of pig production.

When taking these results together, the supplementation of sows with an inorganic-organic mixture could be considered a worthwhile strategy for boosting neonate viability through improved fetal imprinting during gestation and delivering higher levels of mineral nutrients via colostrum.

Final remarks

Although this thesis has aimed to provide strategies for the supplementation of trace minerals as an alternative to traditional pharmacological doses, or even to the use of antibiotics, it should be emphasized that there is no nutritional strategy that could solve management and health outbreaks. Traditionally, antibiotics as well as pharmacological doses of minerals have somehow compensated for weaknesses in farm feed management, temperature conditions, and animal density, among others. Nevertheless, when reducing the use of antibiotics and other antimicrobials, the animal production system must be completely enhanced via a **multifactorial approach** rather than focusing purely on nutritional responsibility. Therefore, these new legislative regulations instead of being an inconvenience for production, could be seen as an opportunity to improve the management of the entire pig industry.

However, as a consideration for future experiments, it would be convenient to evaluate this mineral hydroxychloride source or chelated sources in intermediate or increasing doses in order to identify an inflection point or the optimal dose from which improvements will no longer be observed in the productive performance and health status of animals. Perhaps by using these more available sources, optimal production rates could be achieved with even lower doses than those tested in these experiments.

-Another interesting approach would be to test these sources under a more challenging concept such as the bacterial, parasitic or dietary model (e.g. increasing feed nutrients such as fiber or protein). These models could be used to obtain more detailed knowledge of the mechanisms of action behind these sources and doses of Zn and Cu in critical circumstances, where mineral bioavailability is vital to achieve homeostasis and overcome the challenge.

-As mentioned throughout this dissertation, dietary TM levels and increased populations of resistant bacteria are of major concern with regard to public health. Therefore, the potential long-term impact of feeding with high levels of Cu and Zn or, on the contrary, reduction of feeding levels must be monitored through longitudinal studies under commercial conditions, in order to evaluate the influence of these sustainable strategies.

-An interesting approach in gestating-lactating sows would be to test this blended inorganic-organic source strategy versus a lower dose of completely organic and hydroxychloride minerals through several consecutive breeding cycles to evaluate the medium-long term effects on the reproductive and health indices of hyperprolific sows and their litters, especially in pigs with low birth weights. The benefits for offspring development and the productive longevity of sows might compensate for the use of lower dosages of completely organic and hydroxychloride minerals. Quantification of fecal mineral excretion with these mineral supplementation strategies should be indispensable in the goal of reducing environmental contamination of animal production.

CHAPTER VIII

CONCLUSIONS

Taking the results and interpretations of this dissertation as a whole, it could be concluded that:

A reduction in the supplementation levels of Zn and Cu in pig performance and environmental effects:

1. Reducing Cu doses in feed to a nutritional level (15 mg/kg) for six weeks after weaning impairs the growth of weaned pigs, with at least 1 kg of body weight, as well as reducing their feed efficiency and physiological mineral status, even in the presence of phytase enzyme and particularly with a sulfate source.
2. In fattening pigs, under commercial conditions, reducing the supplementation level of Zn (20 mg/kg) and Cu (15 mg/kg) at nutritional doses in diets with phytase enzyme does not impair performance or carcass composition, but does reduce the excretion of minerals to the environment, mainly with a hydroxychloride source. Since the growing-finishing phase is a long one in swine production, this mineral nutrition strategy would have a significant impact on the sustainability of the pork industry.
3. Although a reduction of TM in the feed may diminish the excessive pressure on the intestinal microbiome community to develop antimicrobial resistance mechanisms, as stated in the present thesis, further long-term commercial studies are needed to evaluate the effect of withdrawing pharmacological TM doses on mechanisms of antimicrobial resistance.

Moderately high levels of Cu in post-weaning diets and their associated mechanisms of action:

4. When pigs are given a choice between nutritional (15 mg/kg) or reasonably high Cu levels (150 mg/kg), they prefer the latter, probably to restore Cu homeostasis,

which is impaired by weaning, and as a positive effect of the neuroendocrine feedback of Cu on feed consumption. Regarding Cu sources, pigs preferred to consume diets supplemented with hydroxychloride source as opposed to sulfate, probably due to the less bitter taste attributed to its chemical solubility differences.

5. Dietary supplementation of Cu (160 mg/kg) clearly modulated intestinal microbial communities towards the reduction of pathogens while increasing saprophytic bacteria. This stable microbiome is in line with the enhanced growth and feed efficiency observed in weaned pigs.

Trace mineral maternal nutrition and neonatal development and programming:

6. Intrauterine competition in hyperprolific litters generates a clear disadvantage in the smallest pigs, which can be partially compensated by supplementing the feed of gestating sows with a blend of inorganic and organic trace minerals in contrast to a totally inorganic supplementation. The smallest pigs born from gestating sows fed the inorganic-organic diet showed an upregulation of genes involved in immune (*TGF-β1*, *FAXDC2*, *HSPB1*), barrier (*ZO1*, *MUC2*, *CLDN15*), antioxidant (*GPX2*, *SOD2*), and digestive (*CCK*, *IGF1R*) function, among others.

CHAPTER IX

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ANNEX

Appendix

Bikker, P., J. van Baal, G. P. Binnendijk, J. T. . van Diepen, L. . Troquet, and A. W. Jongbloed. 2015. Copper in diets for weaned pigs; influence of level and duration of copper supplementation.

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2019	**Training course on statistical techniques with R** *Servei de Estadística Aplicada, UAB*
2019	**English course (B2.2)** *Servei de Llengues, UAB*
2018	**Biological Agents: Risk and Preventive Measures** *Public Health Agency of Canada*

Professional Experience

2017-present	**Member of the Animal Nutrition and Welfare Service** *Universitat Autònoma de Barcelona (Bellaterra)* Collaboration in several research projects (experimental design, farm controls, laboratory analyses, statistical analyses and writing technical reports)
2021	**Member of the Faculty of Veterinary (6 months)** *Universidad Central del Ecuador* Collaboration in research projects and education programs
2016	**M.Sc. Practice (4 months)** *Universidad Politécnica de Madrid* Collaboration in research projects (experimental design, farm controls, laboratory analyses, statistical analyses and writing technical reports)
2016	**M.Sc. Practice (5 months)** *Universidad Complutense de Madrid* Collaboration in microbiology research (laboratory analyses, isolation, and identification of zoonotic pathogens)
2014	**Assistant Professor (6 months)** *Universidad Central del Ecuador* Teaching assistance in Meat Quality and Production area
2014	**Degree practice (6 month)** AVESCA Avícola Ecuatoriana

Fellowships

2018-present	**Pre-doctoral research grant (SENESCYT)** *Secretaria Nacional de Educación Ciencia y Tecnología del Ecuador*
2017	**Scholarship for practice of M.Sc. in Animal Production and Health** *Fundación Premio Arce / Universidad Politécnica de Madrid*

Scientific Publications

Villagómez Estrada, S., Logacho Pilataxi, M., & Vinueza Burgos, C. (2017). Presencia y Resistencia a los Antimicrobianos de serovariedades de Salmonella entérica aisladas en una empresa avícola integrada del Ecuador. Revista Ecuatoriana de Medicina y Ciencias Biologicas, 38(1), 11–24. https://doi.org/10.26807/remcb.v38i1.17

Villagómez-Estrada, S., Blanco, J. L., Melo-Durán, D., Martín, C., Harmanus, C., Kuijper, E. J., & García, M. E. (2019). Detection of Clostridium difficile in the environment in a veterinary teaching hospital. *Anaerobe*, *57*. https://doi.org/10.1016/j.anaerobe.2019.03.011

Villagómez-Estrada, S., Pérez, J. F., Darwich, L., Vidal, A., van Kuijk, S., Melo-Durán, D., & Solà-Oriol, D. (2020). Effects of copper and zinc sources and inclusion levels of copper on weanling pig performance and intestinal microbiota. *Journal of Animal Science*, *98*(5). https://doi.org/10.1093/jas/skaa117

Villagómez-Estrada, S., Pérez, J. F., van Kuijk, S., Melo-Durán, D., Karimirad, R., & Solà-Oriol, D. (2020). Dietary Preference of Newly Weaned Pigs and Nutrient Interactions According to Copper Levels and Sources with Different Solubility Characteristics. *Animals*, *10*(7). https://doi.org/10.3390/ani10071133

Villagómez-Estrada, S., Pérez, J. F., van Kuijk, S., Melo-Durán, D., Karimirad, R., & Solà-Oriol, D. (2021). Effects of two zinc supplementation levels and two zinc and copper sources with different solubility characteristics on the growth performance, carcass characteristics and digestibility of growing-finishing pigs. *Journal of Animal Physiology and Animal Nutrition*, *105*(1), 59–71. https://doi.org/https://doi.org/10.1111/jpn.13447

Villagómez-Estrada, S., Pérez, J. F., van Kuijk, S., Melo-Durán, D., Forouzandeh, A., Gonzalez-Solè, F., D'Angelo, M., Pérez-Cano, F. J., & Solà-Oriol, D. (2021). Strategies of inorganic and organic trace mineral supplementation in gestating hyperprolific sow diets: effects on the offspring performance and fetal programming. *Journal of Animal Science*, *99*(7). https://doi.org/10.1093/jas/skab178

Melo-Durán, D., Pérez, J. F., González-Ortiz, G., Sala, R., **Villagómez-Estrada, S**., Bedford, M. R., Graham, H., & Solà-Oriol, D. (2020). Influence of particle size and xylanase in corn-soybean pelleted diets on performance, nutrient utilization, microbiota and short-chain fatty acid production in young broilers. *Animals*, *10*(10). https://doi.org/10.3390/ani10101904

Melo-Durán, D., Solà-Oriol, D., **Villagomez-Estrada, S**., & Pérez, J. F. (2019). Chapter 20 Enzymes as an alternative to antibiotics: an overview. In *The value of fibre* (pp. 351–371). https://doi.org/10.3920/978-90-8686-893-3_20

Melo-Durán, D, Perez, J. F., González-Ortiz, G., **Villagómez-Estrada, S**., Bedford, M. R., Graham, H., & Sola-Oriol, D. (2021). Growth performance and total tract digestibility in broiler chickens fed different corn hybrids. *Poultry Science*, *100*(8), 101218. https://doi.org/https://doi.org/10.1016/j.psj.2021.101218

Melo-Durán, Diego, Pérez, J. F., González-Ortiz, G., **Villagómez-Estrada, S**., Bedford, M. R., Graham, H., & Sola-Oriol, D. (2021). Maize nutrient composition and the influence of xylanase addition. *Journal of Cereal Science*, *97*, 103155. https://doi.org/https://doi.org/10.1016/j.jcs.2020.103155

Skills and Competences

Language		**Software**	
Spanish	+++++	*Microsoft Office*	+++++
English	++++	*SAS*	++++
Portuguese	++++	*Rstudio*	+++