Effects of High Copper Supplementation from Different Sources on the Productive Performance and Intestinal Microbiota of Broilers and Pigs, and Its Environmental Dimension

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Certify:

That the thesis dissertation entitled "**Effects of high copper supplementation from different sources on the productive performance and intestinal microbiota of broilers and pigs, and its environmental dimension**", presented by Asal Forouzandeh to apply for the degree of Doctor of Veterinary Medicine, 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, on May 25, 2023

Dr. José Francisco Pérez Hernández **Dr. David Solà Oriol** **Dr.Laia Blavi Josa**

Woman, Life, Freedom

زن، زندگی، آزادي

Prelude

This dissertation is an ensemble of my Ph.D. study that started in September 2018 until May 2023 at the Department of Animal and Food Science, Universitat Autònoma de Barcelona, Spain, and was financially supported by Agéncia de Gestió d'Ajusts Universitaris i de Recerca de la Generalitat de Catalunya (Ph.D. grant 2019FI_B 00282).

The study was designed to elucidate the effects of high Cu supplementation from different sources on productive performance and intestinal microbiota of broilers and oxidative status, inflammation, gene abundance, and microbial modulation in pigs. Moreover, the study delves into the effects of Cu on the abundance of genes associated with Cu resistance and the co-selection of antibiotic resistance. The overarching aim of this research was to provide a comprehensive understanding of Cu consumption in animal production, thereby raising awareness about its potential implications and promoting more mindful practices in the market.

As a researcher committed to the pursuit of scientific knowledge, I have undertaken a challenging and ethically complex journey with this Ph.D. thesis. I acknowledge that this study involved animal experimentation, a practice that goes against my personal convictions. Nonetheless, I found myself participating in this research, due to the potential impact of our findings in the animal production section. I firmly believe that the scientific community should continue to work towards minimizing animal suffering and moving away from animal experimentation.

In conclusion, while the decision to conduct this study was not made lightly, I truly hope that the potential benefits, combined with our commitment to ethical research practices, justified the difficult choice to proceed.

Asal Forouzandeh

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Summary

Copper (Cu) has gained significant attention as a replacement for antibiotics due to its antimicrobial properties and ability to enhance animal performance when fed beyond the minimum requirement. However, excessive Cu intake can lead to cellular damage by generating free radicals that cause oxidative stress and liver dysfunction in animals. Furthermore, supplementing animal diets with heavy metals such as Cu increases the likelihood of co-selecting and mobilizing antibiotic-resistance genes, which could eventually lead to the dissemination of antibiotic resistance in humans. Many knowledge gaps still exist regarding the existing sources of Cu in the market and the role that they play in animals' performance, their mechanism of action, and the environmental dimension of high-Cu supplementation. This Ph.D. thesis aimed at investigating the effects of high Cu supplementation from different sources on productive performance and intestinal microbiota of broilers and pigs, and the environmental dimension.

In **manuscript I**, we conducted an experiment to investigate the effects of two sources of Cu, $CuSO_4$ or Cu_2O, at three inclusion levels on the growth performance and gut microbiota of broilers. The study aimed to determine the impact of high-Cu supplementation on broilers challenged with necrotic enteritis by reusing old litter. Our findings revealed that Cu_2O improved body weight and average daily gain as Cu dose inclusion increased from 15 mg/kg to 150 mg/kg. Furthermore, supplementation with 150 mg/kg of Cu from Cu_2O reduced the abundance of *Streptococcaceae* and *Corynebacteriaceae* families, while increasing the abundance of beneficial families such as *Clostridiaceae* and *Peptostreptococcaceae*. We also investigated antimicrobial resistance by analyzing excreta samples and observed that high-Cu supplementation did not alter the antibiotic resistance genes in this study. Our findings suggest that the addition of 150 mg/kg of Cu from Cu_2O can lead to alterations in the gut microbiota, specifically by regulating the bacterial population in the ileum which may provide a potential explanation for the observed improvement in broiler growth performance.

In **manuscript II**, we explored the effects of including high Cu (250 mg/kg) from $CuSO_4$ or Cu_2O in the diet of growing pigs on oxidative stress, inflammation, gene

abundance, and microbial modulation. The study reported that pigs fed diets containing 250 mg/kg of $CuSO_4$ had greater malondialdehyde levels in the liver and higher serum concentrations of tumor necrosis factor-alpha compared to pigs fed the NC diet or the diet with 250 mg/kg Cu from Cu_2O. However, pigs fed diets with high Cu ($CuSO_4$ or Cu_2O) had a greater abundance of genes related to the intestinal barrier function and nutrient transport, as well as a lower abundance of pro-inflammatory genes. Both $CuSO_4$ and Cu_2O supplementation increased the abundance of certain bacterial families and reduced the abundance of certain genera in the colon. Collectively, adding 250 mg/kg of Cu from $CuSO_4$ or Cu_2O regulates genes involved in immunity and growth, and induces changes in the intestinal microbiota. However, Cu_2O induces less systemic oxidation and inflammation compared to $CuSO_4$.

In **manuscript III**, we presented the results of a study, using the same fecal samples from **manuscript II**, based on a novel HT-qPCR metal resistance gene chip in combination with 16S rRNA gene amplicon sequencing and phenotypic resistance typing of *Escherichia coli* (*E. coli*) isolates. The study aimed to investigate the effects of high Cu addition from $CuSO_4$ or Cu_2O on the swine gut bacterial metal resistome and community assembly. The dietary Cu treatments did not significantly affect the bacterial community assembly processes, and the differences in swine gut metal resistome composition were primarily due to variations in bacterial community composition rather than dietary Cu treatments. Even though high dietary Cu intake (250 mg/kg) was selected for phenotypic Cu resistance in *E. coli* isolates, it did not lead to an increase in the prevalence of Cu resistance genes targeted by the HT-qPCR chip. Hence, we conclude that high-Cu supplementation indicates a low risk for co-selection of antibiotic resistance genes via co-resistance or cross-resistance mechanisms, respectively.

Collectively, this thesis demonstrates that high-Cu supplementation, particularly Cu_2O, can improve growth performance and alter the gut microbiota and gene abundance in animals. However, it may also cause oxidative stress and inflammation, which can be mitigated by the source of Cu. Furthermore, the potential risk of co-selection of antibiotic-resistance genes through high-Cu supplementation appears to be insignificant.

Resumen

El cobre (Cu) ha ganado una atención significativa como reemplazo de los antibióticos debido a sus propiedades antimicrobianas y su capacidad para mejorar el rendimiento animal cuando se alimenta más allá de los requisitos mínimos. Sin embargo, la ingesta excesiva de Cu puede provocar daño celular al generar radicales libres que causan estrés oxidativo y disfunción hepática en los animales. Además, la suplementación de la dieta animal con metales pesados como el Cu aumenta la probabilidad de co-selección y movilización de genes de resistencia a los antibióticos, lo que podría conducir a la diseminación de la resistencia a los antibióticos en los humanos. Todavía existen muchas lagunas en el conocimiento sobre las fuentes existentes de Cu en el mercado, el papel que desempeñan en el rendimiento animal, su mecanismo de acción y la dimensión ambiental de la suplementación con alto contenido de Cu. Esta tesis doctoral tuvo como objetivo investigar los efectos de la suplementación con alto contenido de Cu de diferentes fuentes sobre el rendimiento productivo y la microbiota intestinal de pollos y cerdos, así como sobre la dimensión ambiental.

En el **manuscrito I**, se llevó a cabo un experimento para investigar los efectos de dos fuentes de Cu, $CuSO_4$ o Cu_2O, en tres niveles de inclusión sobre el rendimiento de crecimiento y la microbiota intestinal de los pollos. El estudio tuvo como objetivo determinar el impacto de la suplementación con alto contenido de Cu en pollos desafiados con enteritis necrótica al reutilizar la cama vieja. Nuestros hallazgos revelaron que Cu_2O mejoró el peso corporal y la ganancia diaria promedio a medida que aumentaba la inclusión de dosis de Cu de 15 mg/kg a 150 mg/kg. Además, la suplementación con 150 mg/kg de Cu de Cu_2O redujo la abundancia de las familias *Streptococcaceae* y *Corynebacteriaceae*, mientras aumentó la abundancia de familias beneficiosas como *Clostridiaceae* y *Peptostreptococcaceae*. También investigamos la resistencia antimicrobiana analizando muestras de excreta y observamos que la suplementación con alto contenido de Cu no alteró los genes de resistencia a los antibióticos en este estudio. Nuestros hallazgos sugieren que la adición de 150 mg/kg de Cu de Cu_2O puede llevar a alteraciones en la microbiota intestinal, específicamente mediante la regulación de la población bacteriana en el íleon, lo que puede proporcionar

una posible explicación para la mejora observada en el rendimiento de crecimiento de los pollos.

En el **manuscrito II**, se exploraron los efectos de incluir altas dosis de cobre (250 mg/kg) proveniente de $CuSO_4$ o Cu_2O en la dieta de cerdos en crecimiento, sobre el estrés oxidativo, la inflamación, la abundancia génica y la modulación microbiana. El estudio reportó que los cerdos alimentados con dietas que contenían 250 mg/kg de $CuSO_4$ tenían mayores niveles de malondialdehyde en el hígado y concentraciones séricas más altas de factor de necrosis tumoral-alfa en comparación con los alimentados con la dieta NC o la dieta con 250 mg/kg de Cu proveniente de Cu_2O. Sin embargo, los cerdos alimentados con dietas con altos niveles de cobre ($CuSO_4$ o Cu_2O) tuvieron una mayor abundancia de genes relacionados con la función de barrera intestinal y el transporte de nutrientes, así como una menor abundancia de genes proinflamatorios. Tanto la suplementación con $CuSO_4$ como con Cu_2O aumentaron la abundancia de ciertas familias bacterianas y redujeron la abundancia de ciertos géneros en el colon. En conjunto, agregar 250 mg/kg de cobre proveniente de $CuSO_4$ o Cu_2O regula genes involucrados en la inmunidad y el crecimiento, e induce cambios en la microbiota intestinal. Sin embargo, Cu_2O induce menos oxidación e inflamación sistémica en comparación con $CuSO_4$.

En el **manuscrito III**, presentamos los resultados de un estudio basado en el mismo material fecal utilizado en el **manuscrito II**, utilizando un nuevo chip de genes de resistencia a metales HT-qPCR en combinación con la secuenciación de amplicones de genes de ARNr 16S y el tipado de resistencia fenotípica de aislamientos de *Escherichia coli (E. coli)*. El estudio tuvo como objetivo investigar los efectos de la adición de cobre desde $CuSO_4$ o Cu_2O en la resistoma bacteriana del intestino de cerdos y la comunidad bacteriana. Los tratamientos de Cu en la dieta no afectaron significativamente los procesos de ensamblaje de la comunidad bacteriana, y las diferencias en la composición del resistoma de metales del intestino de los cerdos se debieron principalmente a variaciones en la composición de la comunidad bacteriana y no a los tratamientos con Cu en la dieta. Aunque la alta ingesta dietética de Cu (250 mg/kg) seleccionó la resistencia fenotípica al Cu en los aislamientos de *E. coli*, no condujo a un aumento en la prevalencia de los genes de resistencia al Cu dirigidos por el chip HT-qPCR. Por lo tanto, concluimos que la suplementación de alta Cu indica un bajo riesgo de co-selección

de genes de resistencia a antibióticos a través de mecanismos de co-resistencia o cross-resistencia, respectivamente.

En resumen, esta tesis demuestra que la suplementación alta de Cu, particularmente Cu_2O, puede mejorar el rendimiento de crecimiento y alterar la microbiota intestinal y la abundancia génica en animales. Sin embargo, también puede causar estrés oxidativo e inflamación, lo que puede ser mitigado por la fuente de Cu. Además, el potencial riesgo de co-selección de genes de resistencia a antibióticos a través de la suplementación alta de Cu parece ser insignificante.

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GENERAL OVERVIEW

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ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
AMR	Antimicrobial resistance
ARGs	Antibiotic resistance genes
ASV	Amplicon sequence variant
BF	Barrier function
BW	Body weight
CRGs	Cu resistance genes
Ctr1	Cu transport protein 1
Ctr2	Cu transport protein 2
Cu	Copper
E. coli	*Escherichia coli*
EH	Enzyme/hormone
FCR	Feed conversion ratio
GHRH	Growth hormone-releasing hormone
GIT	Gastrointestinal tract
GSH-Px	Glutathione peroxidase
IFN-γ	Interferon-gamma
IGF1	Insulin-like growth factor 1
IL-10	Interleukin 10
IL-12	Interleukin
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
INF-α	Interferon alfa
IR	Immune response
MDA	Malondialdehyde
MDR	Multidrug-resistant

MGEs	Mobile genetic elements
MRGs	Metal resistance genes
NE	Necrotic enteritis
NMDS	Nonmetric multidimensional scaling
NPY	Neuropeptide Y
NT	Nutrient transport
OTU	Operational taxonomic unit
R2A	Reasoner's 2A
RFI	Residual feed intake
ROS	Reactive oxygen species
SOD	Superoxide dismutase
ST	Stress
TNF-α	Tumor necrosis factor-alpha

GENERAL INTRODUCTION

Copper (Cu) was first recognized as an essential nutrient for animals in the 1920s when it was shown that the synthesis of red blood cells in rats requires its presence (Hart et al., 1928). Today, Cu deficiency is known to cause a wide range of problems including anemia, bone disorders, growth depression, depigmentation of fur and feathers, and diarrhea (Leeson, 2009). The beneficial effect of adding Cu in concentrations that exceed the assumed requirement for maximizing the growth of poultry and swine has been previously shown (Hill et al., 2000; Miles et al., 1998). However, Cu has become remarkably useful, after banning antibiotics, due to its antimicrobial properties that improve performance in animals when fed over the minimum requirement (125-250 mg/kg of Cu) (Pang et al., 2009; Vincent et al., 2016).

High dietary Cu may lead to improved growth of monogastric animals through various mechanisms some of which can be attributed to improved fat digestibility (Espinosa and Stein, 2021), and antimicrobial properties (Arias and Koutsos, 2006; Stahly et al., 1980). Interestingly, Cu speciation influences the performance of Cu ions on bacteria with Cu^{+} being more toxic than Cu^{2+} with the elevated antibacterial action of Cu^{+} (Popov et al., 2020; Rensing et al., 2018). Besides, the different solubility and bioavailability of Cu sources may affect intestinal microbiota in a different way (Pang et al., 2009).

Nevertheless, excessive Cu in the organism may exert toxic effects through the formation of free radicals and induced oxidative stress (Gaetke, 2003), changes in lipid profile, and hepatic dysfunction (Sarkar et al., 2011). Besides, bacteria can confer resistance to high levels of Cu with an effect on the evolution of metal and antibiotic resistance via co-selection or co-occurrence (Hasman et al., 2005; Yazdankhah et al.,

2014) with the possibility of entering human and livestock food chain through crops and contaminated water.

Consequently, it is necessary to fully understand the underlying mechanisms which lead to improved performance, and increased toxicity and resistance with high concentrations of Cu.

GENERAL OVERVIEW

1. Emerge, importance and biological functions of Cu

Cu is an ancient metal that has been known to mankind for thousands of years (Konieczny and Rdzawski, 2012). The earliest evidence of Cu smelting, casting, and forging dates to around 5000 BC. However, the process of extracting Cu from more complex ores containing arsenic and lead was not developed until 1000 years later. The first recorded instances of using Cu as a bactericide can be found in Smith's Papyrus, which is considered to be the first and oldest medical document in human history (Dollwet and Sorenson, 1985). Cu was widely used by ancient civilizations for medicinal and hygienic purposes. The Egyptians, Greeks, Romans, and Aztecs all utilized Cu to treat a variety of ailments, such as sore throat and rash as well as to maintain daily hygiene (Konieczny and Rdzawski, 2012). Up to this day, Cu and its alloys continue to be of interest to both researchers and engineers.

Today, Cu is known to be an essential trace element for all living organisms, including bacteria and humans, and is authorized for all species under Directive 70/524/EEC concerning additives in feeding stuff (European Commission, 2003). This trace element makes up a very small percentage (0.00007%) of the Earth's crust and the average adult male body contains around 100 milligrams of Cu (Linder, 2013), however, it has an indispensable role in many physiological functions in the nervous, hematological, cardiovascular, reproduction, and immune systems (Cerone et al., 2000) through specific proteins (Table 1). Most of the biological functions of Cu are thought to be related to its role as a ligand in the active site of metalloenzymes (Hefnawy and El-Khaiat, 2015). Some of the principal enzymes that Cu is involved in include ceruloplasmin

(a plasma glycoprotein that functions as a Cu transport and as an antioxidant), Dopamine-β-monooxygenase (located in noradrenergic neurons and is involved in the conversion of dopamine to norepinephrine), Cytochrome-c-oxidase (the terminal mitochondrial electron carrier), lysyl oxidase (responsible for the oxidative deamination of peptidyl lysine), Cu-Zn-Superoxide dismutase (a cytosolic protein that speeds up the dismutation of superoxide), and Tyrosinase (located in melanocytes and involved in the conversion of tyrosine into melanin). Additionally, Cu is essential for the proper development of antibodies and white blood cells, as well as the production of antioxidant enzymes (Sharma et al., 2005), and its deficiency leads to physiological disturbance.

Despite the abundance of research on Cu, a comprehensive understanding of its role in poultry and swine nutrition is still evolving. Therefore, the following sections will summarize the most vital functions of Cu in poultry and swine.

Table 1. Examples of Cu binding and Cu homeostasis proteins adapted from Kim et al., 2008

Protein	Function
Amyloid precursor protein (APP)	Protein involved in neuronal development and potentially Cu metabolism; cleavage leads to the generation of Aβ peptide that aggregates in senile plaque associated with Alzheimer's disease
Atox1	Metallochaperone that delivers Cu to ATP7A and ATP7B Cu^{1+} transporters
ATP7A	Cu^{1+}-transporting P-type ATPase expressed in all tissues except liver
ATP7B	Cu^{1+}-transporting P-type ATPase expressed primarily in the liver
Carbon monoxide dehydrogenase to acetyl-CoA synthase	*Moorella thermoacetica* bifunctional enzyme; reduces CO2 to CO with subsequent assembly of acetyl-CoA
Ceruloplasmin	Serum ferroxidase that functions in Fe^{3+} loading onto transferrin
Coagulation factors V and VIII	Homologous pro-coagulants present on the surface of platelets, where they nucleate the assembly of multiprotein proteolytic complexes involved in blood coagulation
CCS	Metallochaperone that delivers Cu to Cu/Zn SOD
CopZ	*Archaeoglobus fulgidus* [2Fe-2S] and Zn^{2+} -containing Cu chaperone
Cox17	Metallochaperone that transfers Cu to Sco1 and Cox11 for cytochrome oxidase Cu loading in mitochondria
Ctr1	High-affinity Cu^{1+} transporter involved in cellular Cu uptake
Cu/Zn SOD (SOD1)	Antioxidant enzyme, catalyzes the disproportionation of superoxide to hydrogen peroxide and dioxygen
Cytochrome *c* oxidase	Terminal enzyme in the mitochondrial respiratory chain, catalyzes the reduction of dioxygen to water
Dopamine β-hydroxylase (DBH)	Oxygenase, converts dopamine to norepinephrine
Ethylene receptor (ETR1)	Member of a plant receptor family that uses a Cu cofactor for ethylene binding and signaling
Hemocyanin	Oxygen transport protein found in the hemolymph of many invertebrates such as arthropods and molluscs
Hephaestin	Transmembrane multi-Cu ferroxidase; involved in iron efflux from enterocytes and macrophages
Glucose oxidase	Pentose phosphate pathway oxidoreductase that catalyzes the oxidation of D-glucose into D-glucono-1, 5-lactone and hydrogen peroxide
Laccase	Phenol oxidase involved in melanin production
Lysyl oxidase	Catalyzes formation of aldehydes from lysine in collagen and elastin precursors for connective tissue maturation
Metallothionein	Cysteine-rich small-molecular-weight metal-binding and detoxification protein
Peptidylglycine-α-amidating mono-oxygenase (PAM)	Catalyzes conversion of peptidylglycine substrates into α-amidated products; neuropeptide maturation
Prion protein (PrP)	Protein whose function is unclear but binds Cu via the N-terminal octapeptide repeats
Steap proteins/Fre1/Fre2	Family of metalloreductases involved in Fe^{3+} and Cu^{2+} reduction
Tyrosinase	Monophenol mono-oxygenase; melanin synthesis
XIAP	Inhibitor of apoptosis through binding and catalytic inhibition of several caspases

1.1. Immune function

The host must have a functioning immune system to defend against pathogen infections. This function becomes crucial in harsh conditions, such as high animal density or poor sanitation. Additionally, young animals like piglets after weaning and chickens with underdeveloped intestines and immune systems, are extremely susceptible to infection by microorganisms. Cu plays a crucial role in the improvement and maintenance of the immune system, which relies on Cu for various functions (Hefnawy and El-Khaiat, 2015). It is vital for the growth and functioning of T and B cells, neutrophils, and macrophages (Sorenson, 1989). Cu deficiency affects the immune system by altering the levels of enzymes such as cytochrome C oxidase and superoxide dismutase (Prohaska, 1983). Low levels of cytochrome C oxidase cause a decrease in the respiratory burst in neutrophils, leading to a reduction in immune function (Segal and Meshulam, 1979). When pigs are exposed to pathogens or non-pathogenic antigens, the immune system is activated, resulting in the release of cytokines such as tumor necrosis factor α, interleukin-1, and interleukin-6 (Al-Sadi et al., 2009). Adding Cu to pig diets resulted in lower levels of tumor necrosis factor α in blood serum and increased activity of superoxide dismutase in weanling pigs (Espinosa et al., 2020; Gonzales-Eguia et al., 2009). The immune system benefits of high Cu diets in pigs may be indirect due to Cu's ability to inhibit bacterial growth and therefore reduce inflammation caused by pathogens (Espinosa et al., 2019). Indeed, many animal studies have shown that Cu has an impact on various aspects of the immune system indirectly (Arthington and Havenga, 2012; Espinosa et al., 2019; Schuschke et al., 2016). Therefore, within the framework of this thesis, we will provide new insight into the influence of Cu on the diet of swine and poultry.

1.2. Gut microbiota modulation and antimicrobial function

The intestinal microbiota of mammals and chickens is a significant metabolic organ that contributes to immune competency, feed digestibility, and nutrient absorption (Apajalahti, 2005; Schokker et al., 2015). Research has shown that the addition of Cu to poultry and swine diets can modulate this bacterial community in the gastrointestinal tract (GIT) and/or populations of (potential) pathogens (Table 2). Some of these changes can be challenging to understand and may conflict with established ideas about a desirable composition of the microbiota.

Table 2. Mechanistic insights of Cu effects on the gut environment investigated by recent livestock studies, adapted from Broom et al., 2021

Species	Mineral Supplementation Level and Source	Key Mechanistic Insights	Reference (s)
Pig	0–300 mg/kg (CuSO4 or HCl)	• Reduction in notable fecal butyrate-producing bacteria, lower fecal protein, and carbohydrate-related metabolites, decreased serum TNF-α, and higher total antioxidant capacity. • Altered GIT microbiota, microbial energy, and protein metabolism, increased *E. coli* and multidrug resistant isolates.	(F. Zhang et al., 2019) (Y. Zhang et al., 2019) (Villagómez-Estrada et al., 2020)
Chicken	1.7–250 mg/kg (Nanoparticles, CuSO4 or Cu acetate)	• Modified cecal bacterial community. • Decreased duodenal lesion score following mixed Eimeria challenge. • Reduced *S. Typhimurium* colonization of cecal tonsils following oral challenge.	(Yausheva et al., 2018) (Santos et al., 2020) (Leyva-Diaz et al., 2021)

Studies on the effects of Cu on the gastrointestinal environment of poultry have shown that increased dietary Cu can improve the similarity of bacteria in the ileal mucosa of broiler chickens and influence intestinal morphology and immune responses either directly or indirectly (Arias and Koutsos, 2006). This alteration is believed to reduce the recruitment and infiltration of intestinal lymphocytes and enhance nutrient absorption (Arias and Koutsos, 2006; Bunch et al., 1965; Hawbaker et al., 1961). Cu

supplementation can reduce the colonization of *Salmonella Typhimurium* in broiler chickens and increase the presence of the *Lachnospiraceae* genus in the caeca (Leyva-Diaz et al., 2021). *Lachnospiraceae* are important for short-chain fatty acid production but have also been linked to intestinal diseases (Vacca et al., 2020). These findings suggest the potential of Cu to influence the gut microbiome and immune-related features in chickens.

In the porcine model, some studies have revealed that high levels of Cu in the diet can reduce the diversity of gut bacteria in pigs, with a decrease in lactic acid bacteria and an increase in coliforms (potential pathogens) (Højberg et al., 2005; Namkung et al., 2006). Some bacterial genera that produce butyrate, a beneficial short-chain fatty acid, were also reduced with high levels of dietary Cu. It was suggested that the reduction in lactic acid bacteria could enhance the availability of nutrients for the host organism, and the control of coliforms, which potentially contain pathogens, could be beneficial. On the other hand, another study found that high levels of dietary Cu had no effect on the diversity of gut bacteria in pigs but was found to decrease the presence of prominent butyrate-producing bacteria, while it increased the abundance of *Streptococcus* and *Fibrobacter* (F. Zhang et al., 2019). Reduced fecal metabolites associated with protein and carbohydrate digestion, absorption, and metabolism were observed in the group of pigs fed a high Cu diet, suggesting enhanced digestive utilization of dietary nutrients.

The antimicrobial attributes of Cu have been widely acknowledged and recorded over time, with its first mention dating back to an Egyptian papyrus from 2600-2200 BC (Dollwet and Sorenson, 1985). In the modern era, it has been used in hospitals and other healthcare settings as a way to prevent the spread of infectious diseases (Schmidt et al., 2013, 2012). Cu surfaces can kill a wide variety of microorganisms, including bacteria,

viruses, and fungi, and studies have shown that using Cu surfaces in hospitals can lead to significant reductions in infection rates. Three mechanisms are proposed to explain Cu's antibacterial activity (Konieczny and Rdzawski, 2012):

1) Reactive oxygen species (ROS) generation: an increased concentration of Cu within a cell can lead to oxidative stress, causing an imbalance between the activity of ROS and the biological capacity for detoxification and damage repair. This results in the generation of hydrogen peroxide and the initiation of Fenton's reaction.
2) Membrane damage: when there is an excess of Cu, the membrane integrity of microorganisms can be compromised, resulting in the leakage of specific nutritional elements such as potassium and glutamate from the cell. This can lead to desiccation and ultimately to the death of the cell.
3) Protein denaturation: although Cu is required for many protein functions, an excess of Cu can combine with proteins that do not require its presence, leading to improper bonding. This improper bonding can lead to the atrophy of protein functions and/or the decay of proteins into dysfunctional components.

These mechanisms all contribute to the antibacterial properties of Cu and explain why Cu and its alloys are effective in reducing the survival and transmission of a wide range of bacteria.

Since the European Union (Regulation 1831/2003/EC) banned antibiotics as growth promoters in food-producing animals in 2006 (Castanon, 2007), considerable efforts have been made to discover alternatives for antibiotics that can produce comparable outcomes by altering the intestinal microflora, augmenting immune system performance, and stimulating animal growth (Sabry et al., 2021). Potential antimicrobial

and growth-promoting properties of Cu, positioning it as a promising alternative for antibiotic growth promoters. Indeed, the administration of 175 mg/kg $CuSO_4$ resulted in a decrease in coliforms count and thus likely pathogens present in the large intestine of pigs (Højberg et al., 2005). This reduction in coliforms may be attributed to various other mechanisms, including the inhibition of specific pathogens, enhancement of the animal's resistance to pathogen adhesion and invasion, and the prevention of the production of toxins by pathogens (Carlson et al., 2004). Studies have investigated the antimicrobial properties of Cu through in-vitro assays (Pang and Applegate, 2007) and in-vivo assays using plate counting (Mei et al., 2009; Namkung et al., 2006). However, the effects of Cu are not lethal to all intestinal bacteria species. Some bacterial groups, particularly pathogens, are suppressed by Cu, while others increase in response (Broom et al., 2021).

The advantages of providing monogastric livestock with Cu in amounts that are higher than what is nutritionally required or through pharmacological means are evident, but the underlying processes responsible for these effects are not fully understood. Given that various sources of Cu have varying solubility and chemical properties within the GIT, they might impact the intestinal microflora differently. Considering the superior inhibitory potential of Cu^{+} compared to Cu^{2+} ion in controlling both gram-positive and gram-negative microorganisms (Saphier et al., 2018), it is crucial to investigate the appropriate dosages and sources of Cu that can effectively manage microbial pathogens while minimizing their adverse effects.

2. Digestion and absorption

The assessment of Cu and Zn digestibility is challenging because the body's homoeostatic regulation interferes and restricts the absorption of these minerals beyond the animals' requirements (Lebel et al., 2014). For growing pigs, the digestibility of Cu ranges from 30% to 55%, and its low digestibility is attributed to the antagonism between Cu and other microminerals (Richards et al., 2010). Additionally, the low pH in the stomach can cause inorganic salts of dietary Cu to dissociate, reducing its digestibility (Underwood and Suttle, 1999). In the small intestine, as the pH increases, Zn and Cu may become trapped in insoluble hydroxide precipitates, making these minerals unavailable for absorption (Powell et al., 1999). Furthermore, the source of Cu in the diet also affects its digestibility in pigs (Lebel et al., 2014).

Cu is primarily absorbed in the upper GIT, especially in the duodenum, but some Cu is also absorbed in the stomach (van Campen and Mitchell, 1965). The absorption of Cu in animals is influenced by various factors such as the animal's physiological stage, the amount of Cu in their diet (Jenkins and Hidiroglou, 1989), and its interactions with compounds like phytate, ascorbic acid, fiber, and tannins, which can form complexes with Cu as well as other trace elements (Cousins, 1985). Cu exists in two forms of valency, depending on its oxidation state. The majority of dietary Cu is in the Cu^{2+} form, but in order to be absorbed, it must be reduced to Cu^{+}, which is catalyzed by a Cu-reductase enzyme expressed by glands at the brush border (Georgatsou et al., 1997). Once dietary Cu^{2+} is reduced to Cu^{+}, it enters the enterocyte through the Cu transport protein 1 (Ctr1) and crosses the apical membrane (Figure 1). Ctr1 is the main transporter for Cu in enterocytes due to its strong affinity for Cu. If Cu is in excess of the requirement, the amount of Ctr1 in the apical membrane decreases via degradation in endosomal

compartments (Hill and Link, 2009). Cu transport protein 2 (Ctr2) and divalent metal transporter (DMT1) are other Cu transporters involved in Cu uptake, but their affinity for Cu is lower than that of Ctr1 (Zhou and Gitschier, 1997). Therefore, Ctr1, Ctr2, and DMT1 are the transport proteins that specifically increase cellular Cu concentration when the body requires Cu.

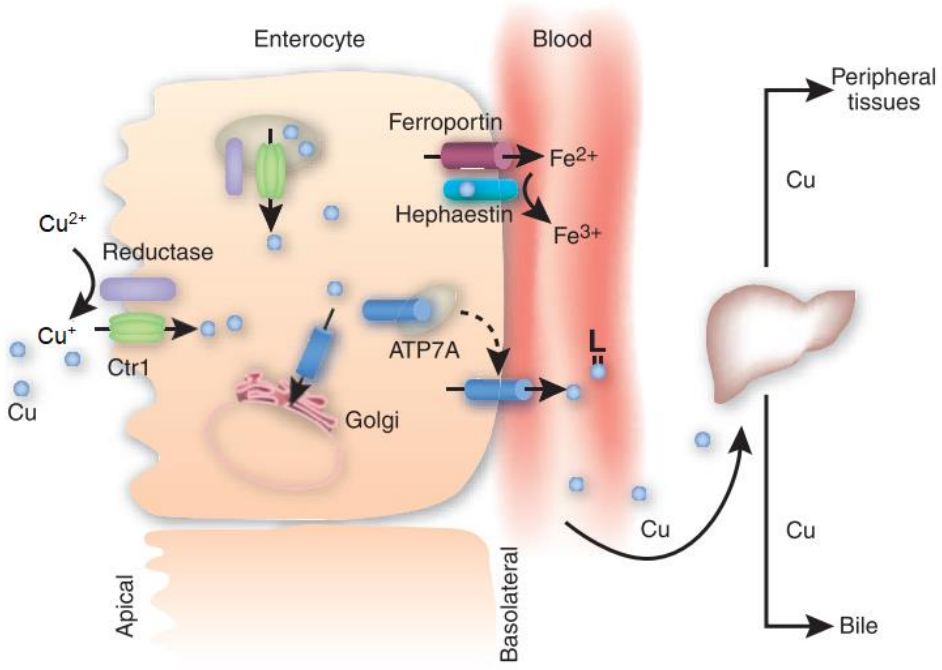

Figure 1. Intestinal Cu absorption and peripheral distribution adapted from Kim et al., 2008. The high-affinity Cu^{+} transporter Ctr1 is present on the apical membrane of intestinal epithelial cells and inside intracellular vesicles. Cu^{2+} is converted to Cu^{+} by a potential metalloreductase to enable its transport by Ctr1. The mobilization of Cu^{+} from endosomal compartments may also involve Ctr1 and a metalloreductase. Cu^{+} is directed to the secretory compartment to be loaded onto Cu-dependent enzymes or exported out of the basolateral membrane via the Cu^{+}-transporting P-type ATPase ATP7A. The portal vein carries Cu through the bloodstream to the liver. Cu is transported to peripheral tissues through the systemic circulation bound to one or more unidentified ligands (L). Excess Cu is excreted in bile.

When Cu^{+} is taken up from the apical membrane, it is then transferred to chaperone proteins (Vonk et al., 2008). The chaperone proteins play a vital role in regulating the Cu concentration in the body and are associated with specific metalloenzymes and other

proteins that contain Cu (Hill and Link, 2009). One of the chaperone proteins is responsible for delivering Cu^{+} to the antioxidant enzyme Cu/Zn-superoxide dismutase. Another chaperone protein, known as COX17, transports Cu^{+} to the mitochondria where it is used by cytochrome C oxidase for energy transfer from NADH or FADH2 to ATP (Cater and Mercer, 2006). In addition to these, another chaperone protein, ATOX1, delivers Cu through the cytosol to the Golgi apparatus of intestinal cells (Lutsenko et al., 2007). Cu is later transferred to ATP7A (Kim et al., 2009). ATP7A not only transports Cu to the basolateral membrane, but it also helps to prevent Cu toxicity by sequestering excess Cu (Axelsen and Palmgren, 1998). Once at the basolateral membrane, Cu^{+} is converted to Cu^{2+} via a Cu oxidase, and then released into the interstitial space.

The absorption rate of Cu can be affected by the Cu status of the organism, and it has been observed that Cu digestibility may be higher in Cu-deficient animals (Davis and Mertz, 1987). In Cu-deficient animals, the synthesis of Cu transport proteins is increased, and a Cu-ATPase pump is used to transfer Cu across the basolateral membrane to the extracellular fluid (Davis and Mertz, 1987), thereby aiding in Cu absorption. In contrast, when animals have adequate Cu concentration, the liver synthesizes metalloenzymes and stores Cu for future use, and the amount of Cu transport proteins required for uptake is low. On the other hand, if the dietary intake of Cu exceeds the body's requirement, enterocytes generate a protein called metallothionein that has a high sulfhydryl content, which binds to the freely ionized Cu. This leads to a decrease in Cu absorption, which helps to prevent Cu toxicity (Carlson et al., 1999; Cousins, 1985).

After Cu is absorbed from the small intestine, it enters the hepatic portal vein and binds to albumin and transcuprein (Linder, 2013) for transport to the liver, where it is taken up by hepatocytes (Figure 2). Cu reductase is used to convert Cu^{2+} to Cu^{+}, which is

then transported across the hepatocyte cell membrane by the Ctr1 protein. To transport Cu from the liver to peripheral tissues, Atox1 delivers Cu to the transmembrane Golgi complex, where it is transferred to ATP7B protein (Kim et al., 2009). The Cu bound to ATP7B is then used to produce Cu-containing proteins that are exported from the liver. Ceruloplasmin is the major protein carrier for the export of Cu from the liver to target organs and contains most of the Cu in serum (Roeser et al., 1970). In pigs, there are two forms of ceruloplasmin (Milne and Matrone, 1970), ceruloplasmin I and II, with ceruloplasmin I having a greater Cu content and specific enzymatic activity. Newly born piglets have high concentrations of liver Cu with ceruloplasmin II as the predominant form, but as they grow older, the concentration of ceruloplasmin I increase while ceruloplasmin II remains constant (Milne and Matrone, 1970).

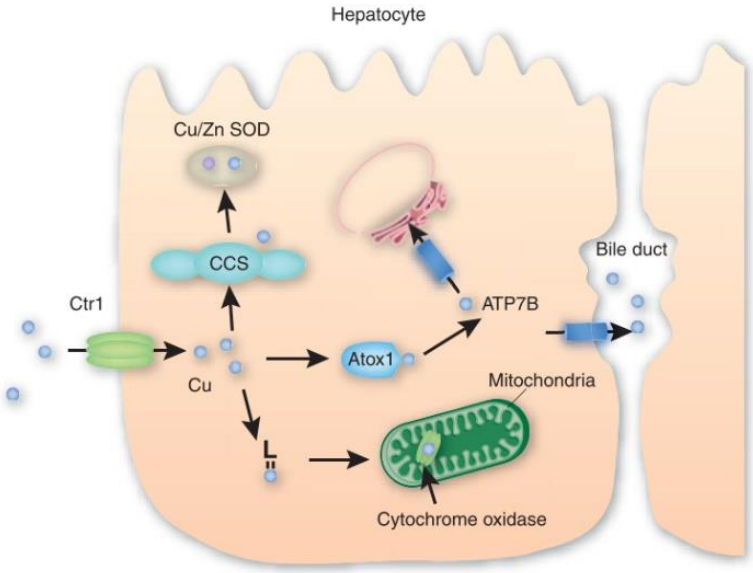

Figure 2. Cu import and trafficking in hepatocytes adapted from Kim et al., 2008. Cu^{+} is transported through the plasma membrane by Ctr1 and delivered to Cu/Zn SOD1 via CCS. The ATP7B Cu^{+}-transporting P-type ATPase at the secretory apparatus and Atox1 at the bile canalicular membrane are involved in directing Cu^{+} to its destination. The movement of Cu to the mitochondria may involve unknown intracellular ligands (referred to as L).

3. Antagonisms and interactions with other nutrients

Another important aspect to consider regarding Cu nutrition is that the proportion of available Cu can be quite variable, and such variation may well be impacted by several dietary compounds which interfere with Cu absorption. Consequently, it is necessary to recognize these factors when formulating diets for animals to ensure that they receive adequate levels of Cu without compromising its absorption and utilization. Classical examples of mineral interactions include dietary Zn, Fe, S, and Mo, as well as phytate interaction. Zinc shares a close chemical and physiological relationship with Cu. It plays a vital role in the activation of various metalloenzymes, including superoxide dismutase, which requires both Cu and Zn as components (O'Dell, 2000). Consuming high amounts of Zn can increase the need for Cu (Davis and Mertz, 1987) as it prompts the production of intestinal metallothionein, a protein that binds to Cu and reduces its absorption (Cousins, 1985). Consequently, a high Zn intake can lead to clinical symptoms of Cu deficiency (Esparza Gonzalez et al., 2005; Hill et al., 1983). When the diet contains high levels of Fe, Cu absorption can be reduced, resulting in Cu deficiency (Hedges and Kornegay, 1973). This is because Fe and Cu compete for absorption sites in the intestinal mucosa, and therefore have antagonistic effects (Hedges and Kornegay, 1973). Additionally, the interference of Fe in Cu absorption is due to the formation of ferrous sulfide complexes (Collins et al., 2010). Cu forms insoluble complexes with the sulfide portion of the complex (Abrahams and Thornton, 1984). Mo is necessary to facilitate the release of Fe from the intestinal mucosa. When there is a deficiency of Mo or antagonism by other minerals, it can result in anemia (Seelig, 1972). An excess of either Cu or Mo can cause a deficiency of the other, leading to symptoms of hypochromic microcytic anemia that are identical to those of an uncomplicated Fe deficiency (Leeson,

2009). The use of pharmacological levels of dietary Cu for promoting growth of animals in commercial practice has raised concerns about its compatibility with exogenous phytase enzymes. High levels of dietary Cu can cause a decrease in the solubility of the phytic acid complex (Leeson, 2009). The reason is phytic acid binds with dietary cations, including Cu, making them unavailable for digestion and absorption (Martin and Evans, 1986). Supplementing phytase can increase Cu absorption by releasing Cu from phytic acid (Adeola, 1995). However, microbial phytase may decrease Cu availability by releasing significant amounts of Zn that are bound to phytate (Aoyagi and Baker, 1995).

Overall, Cu's ability to exist in multiple valence states can result in interactions with other minerals and nutrients, impacting not only Cu metabolism but also that of other minerals and chelates. Hence, employing various strategies such as modifying the form of Cu to influence its absorption and interaction with other minerals, as well as monitoring Cu intake to prevent overloading the system, can help reduce Cu interactions. Adopting these approaches can aid in mitigating the negative impacts of Cu and promoting better mineral balance for overall animal health and productivity.

4. Deficiency and Excess

As mentioned in the previous paragraphs the importance of Cu in animal health and disease is well documented. Therefore, it is not surprising that animals develop critical dysfunctions in case of Cu deficiency or surplus. Cu deficiency as well as Cu toxicity can both occur naturally and may result in decreased animal productivity, reproduction, organ dysfunctions, and the development of pathological lesions (Kumar et al., 2015; Murawski et al., 2006). The effects and their severity are determined by various factors including the timing of Cu deficiency during reproduction and development, the degree

of Cu deficiency, and the particular animal species involved (Mason et al., 1989). Cu deficiency primarily affects several major organ systems, including the blood and hematopoietic system, cardiovascular system, connective tissue and bone, nervous system, and immune system (McArdle and Ralph, 2001). Indeed, studies have shown that a deficiency in Cu can result in various negative effects on pigs. Cu deficiency resulted in reduced levels of serum ceruloplasmin, which lead to anemia (Ragan et al., 1969). Cu deficient diet can lead to bone abnormalities and leg conditions with varying degrees of crookedness in pigs due to the deficiency in monoamine oxidase which is required for cartilage formation (Lahey et al., 1952; Lorenzen and Smith, 1947). Studies have shown depigmentation, failure of hair keratinization, and cardiovascular disorders as symptoms of Cu deficiency in pigs (Graham, 1977; Savage et al., 1966). More than 60% of pigs fed diets deficient in Cu have died from coronary artery disease (Coulson and Carnes, 1963), which is characterized by intimal lesions in the muscular arteries of Cu-deficient pigs (Carnes et al., 2006).

Undoubtedly, all the typical signs resulting from Cu deficiency are of significant concern, but the alteration in immune response and the subsequent impact on growth performance, particularly in challenging sanitary conditions, is perhaps the most significant physiological and productive repercussion.

Cu is essential for the proper development and function of immune cells, such as T and B cells, neutrophils, and macrophages (Miller et al., 1979; Sorenson, 1989). When there is a deficiency of Cu, it can negatively impact the immune system due to a lack of cytochrome C oxidase and superoxide dismutase (Prohaska, 1983). Neutrophils experience a decrease in immunological function as a result of the impairment of the respiration burst due to low cytochrome C oxidase concentration (Segal and Meshulam,

1979). The typical clinical signs and symptoms observed in pigs with Cu deficiency are commonly linked to the role of Cu as a constituent of metalloenzymes necessary for various metabolic reactions, including hemoglobin formation, cartilage formation, cellular respiration, and keratinization, among others, as noted by Lahey et al. (1952). When diets for all animal species lack sufficient Cu, growth performance and feed intake are reduced, however, an unusual leg condition develops particularly in the case of pigs (Teague and Carpenter, 1951). For broilers, low to deficient levels of dietary Cu have been linked to subtle changes in the saturation of body lipid reserves and cholesterol metabolism, which can cause hypertriglyceridemia, hypercholesterolemia, and anemia (Kaya et al., 2006).

By contrast, an overabundance of Cu can also have negative physiological effects. If the amount of Cu in the diet exceeds the animal's requirement, Cu may accumulate in critical organs, such as the liver. This accumulation can cause an increased concentration of unbound free ionized Cu, which is a potent oxidant and may lead to haemolysis or breakdown of red blood cells (NRC, 2012). Feeding pigs with a diet containing more than 250 mg/kg of Cu for a prolonged period can lead to Cu toxicity, which is characterized by the destruction of red blood cells causing jaundice and necrosis (NRC, 2012; Jacela et al., 2010). It is worth noting that pigs are more resistant to Cu toxicity compared to ruminants (Goff, 2018). However, feeding Cu in excess (300-500 mg/kg) for an extended period can still cause liver damage since it is the primary organ where Cu is stored following its absorption (Gaetke et al., 2014). The addition of 750 mg/kg of diet to growing pigs led to elevated concentrations of Cu and aspartate transaminase in the serum (Suttle and Mills, 1966). The increase in serum aspartate transaminase levels is an indication of tissue damage in organs where it is present in high levels, such as the

liver and kidney (Ellingsen et al., 2007). Gao et al. (2020) provided an extensive review of the consequences of prolonged Cu feeding in pigs. These include oxidative damage to nerve cells, which weakens feeding signal transmission, as well as chronic gastritis and ulcers. In addition, the activities of some digestive enzymes are reduced, the rate of digestion is slowed, and chyme transit time in the intestine is prolonged, which as a result, pigs to experience enhanced satiety and inhibited food intake. With various breeds of chicks, graded levels of supplemental Cu chloride were found toxic with 640 mg for weight gain and 780 mg for feed utilization.

A high Cu diet can also cause oxidative stress in both diets and the body, promoting lipid peroxidation in cell membranes (Bremner, 1998). This can degrade unsaturated fatty acids and reduce energy in diets, which may have a negative impact on the growth performance and health of pigs (Lykkesfeldt and Svendsen, 2007). The extent of oxidative stress can differ and may be affected by the type of diet and the source of Cu. To assess the level of peroxidation in an animal's body, one effective approach is to use malondialdehyde (MDA) as a biomarker of oxidative stress (Fry et al., 2012).

It is important to note that providing pharmacological levels of Cu (300 mg/kg) in the diets of pigs and poultry exceeds their requirement by more than 40 times (8 mg Cu/kg for broilers; NRC, 1994 and 5-6 mg Cu/kg in case of piglets; NRC, 2012). After an extended period of overdose, it is possible for homeostasis mechanisms to ultimately overcome the toxic effects of excess Cu. However, the chemical nature of Cu may be an important consideration in mineral toxicity, as highly absorbable sources of Cu administered over a prolonged period have been found to increase the risk of toxicity (NRC, 2012).

5. Current legal practices in poultry and swine production

Cu is a crucial trace element in the physiological functioning of animals (Figure 3), but it cannot be stored in the body, so a regular dietary supplement is needed. Additionally, feed ingredients usually do not contain sufficient levels of Cu.

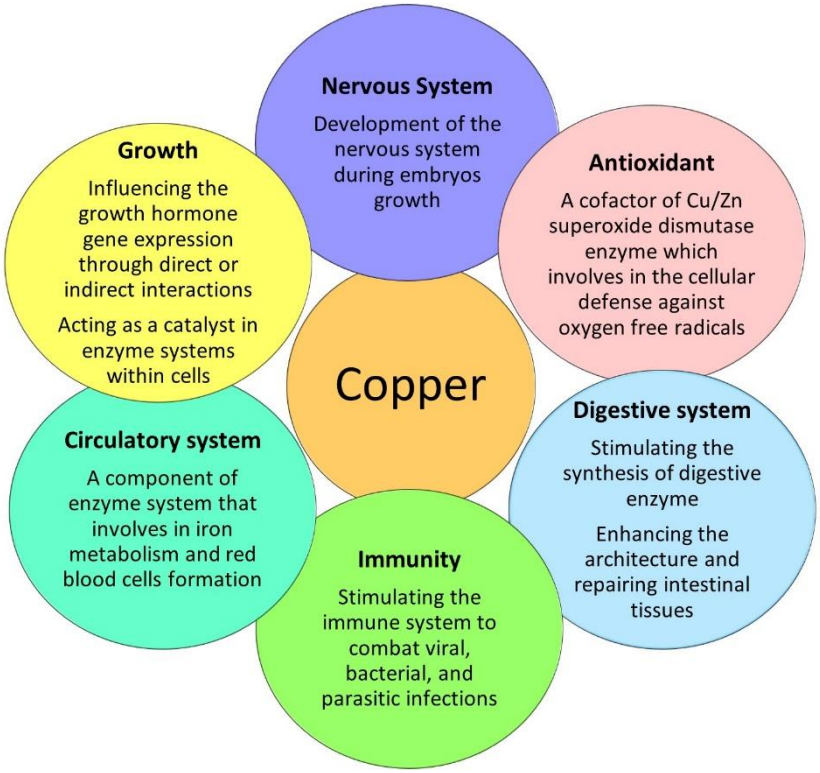

Figure 3. The importance of Cu for the physiological functioning of the animal body adapted from Sabry et al. (2021).

Hence, the commercial diet must provide the required amount of biologically available Cu supplement, which depends on the physical and chemical characteristics of the supplement (European Commission, 2003). While there may be slight variations across organizations, the dietary requirements for growing pigs and chickens typically range between 3-15 mg/kg of feed (Table 3). However, the maximal allowed level of Cu is corresponding to 150 mg/kg Cu from lactation until 4 weeks after weaning, 100 mg/kg

between the 5th and 8th weeks after weaning, and 25 mg/kg after the 8th week (regulation EU 2018/1039).

Table 3. Copper requirements and recommendation (mg/kg) for swine and poultry, set up by different scientific bodies

	Swine				Poultry		
	Piglets	Growing-finishing	Sow gestating	Sow lactating	0-14 days	15-36 days	37-44 days
NRC[1], US	5 - 6	3 - 4	5	5	5	8	8
FEDNA[2], Spain	8 - 15	8 - 13	10 - 15	10 - 15	7	6	4
Rostagno[3], Brazil	13 - 17	6 - 10	13	13	-	-	-
INRA, France	10	10	10	10	-	-	-
BSAS[4], UK	6	6	6	6	-	-	-

[1]NRC (Poultry, 1994; Swine, 2012) 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.
[3]Rostagno (2017) 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.
[4]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.

5.1. Requirements and recommendations

The term "nutritional requirement" refers to the minimum amount of certain nutrients that an organism needs to maintain health, support growth, optimize production, and achieve reproductive success. Trace element requirements are typically determined through dose-response experiments, as there is often insufficient experimental data available for a factorial approach (European Commission, 2003). However, studies on trace element requirements can yield variable results due to the many factors that can influence the outcome of experiments, such as the criteria used to evaluate results, the composition of the basal diet, and the animal breed being studied. Due to the challenges involved in accurately determining trace element requirements, several national scientific organizations provide recommendations for trace element intake rather than establishing specific requirements. These recommendations typically include a safety margin to ensure that the animal's needs

are met, even under high-performance conditions. By providing recommendations, these organizations aim to promote safe and effective trace element supplementation, while minimizing the risk of adverse effects associated with over-supplementation. Hence, the requirements for trace elements are typically viewed as the baseline necessary to address a deficiency, rather than to enhance efficiency or resilience.

Although modern animal breeds used for food production exhibit greater productive performances compared to older breeds, Cu supplementations are typically formulated based on the NRC guidelines. Regarding poultry, there is a scarcity of data on Cu requirements as given by NRC (1994), and the values provided for all species are established on the findings of only five studies. Additionally, during the immunological stress in poultry Cu requirements may increase. In commercial swine production, during periods of stress such as the initial days following weaning, or especially when piglets are weaned at very young ages, their natural appetite is often suppressed or even absent. Therefore, providing feed with low nutritional content would not be suitable to meet the physiological requirements of the pigs. Overall, it should be taken into account that the requirements and recommended values may vary depending on the genetic crossbreed, health, and immune status of animals.

5.2. *Sources and chemical characterization*

Cu supplementation is necessary for most species due to the low Cu content found in certain feedstuffs, which may not meet the recommended requirements and may have varying bioavailability. In commercial monogastric diets, there are several available sources of Cu that are chosen based on their solubility characteristics, mineral content

percentage, relative bioavailability, and economic value. These sources are typically categorized as either inorganic or organic (Table 4).

Table 4. Characteristics of common Cu source adapted from Ph.D. thesis of Sandra Villagómez Estrada (2021)

Source	Compound	Chemical formula	Element content, %	Relative bioavailability, %
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

Usually, inorganic sources consist of compounds such as oxides, sulfates, chlorides, and carbonates, which are ionic salts bonded to inorganic materials. The relative bioavailability of Cu in the diet refers to the amount of ingested Cu that has been chemically absorbed and can be effectively used by the animal for growth and maintenance purposes (Baker and Ammerman, 1995). The reference standard most frequently employed to assess the bioavailability of Cu from various sources is cupric sulfate pentahydrate (Lonnerdal et al., 1985). It is important to note that relative bioavailability does not indicate the percentage of mineral absorbed or retained by an animal, as the absorbed and retained mineral is typically less than 50% of the intake (NRC, 2012). True bioavailability, on the other hand, refers to the proportion of an ingested nutrient that is absorbed, transported to its site of action, and utilized by the body in its physiologically active form (O'Dell, 1983). To determine the relative bioavailability of Cu, liver, bile, and gall bladder are typically collected and Cu concentrations are measured (Aoyagi and Baker, 1995; Cromwell et al., 1989).

Additionally, indicators of Cu status such as plasma Cu concentrations, metalloproteins, and metalloenzymatic activities (e.g., ceruloplasmin, cytochrome C oxidase, and Cu-superoxide dismutase) can be used (Kegley and Spears, 1994). While it may seem that water-soluble sources are easily absorbable and therefore more suitable for animal physiology, doubts about their actual effectiveness have arisen from published animal studies that compare different mineral sources. According to Spears (1996), organic and inorganic forms are metabolized differently following absorption.

Despite the variety of Cu sources, the feed industry still opts for $CuSO_4$ due to its cost-effectiveness compared with other sources of Cu (Shelton et al., 2008). $CuSO_4$ is highly soluble in water and acidic solutions and studies have demonstrated its ability to improve growth performance and gut health in chickens and weanling pigs (Samanta et al., 2011; Pérez et al., 2011). Nonetheless, this solubility feature renders $CuSO_4$ susceptible to interacting with other diet components, such as phytic acid and other minerals (Ren et al., 2021; Santos et al., 2015), potentially hindering their absorption and availability. Indeed, the use of pharmacological concentrations of $CuSO_4$ has led to antagonisms with other dietary components (Wang et al., 2007) and raised environmental concerns due to the high excretion of Cu in feces (Zhao et al., 2014). Due to the negative impacts of supplemented Cu, it is necessary to re-evaluate the commercially available sources to reduce and optimize mineral supplementation.

Organic sources of Cu, also known as "chelates", are another category. In this category, the soluble metal salt is covalently bonded to a protein, peptide, amino acid, or organic acid molecule (AAFCO, 2002). This chemical structure provides a protective layer, making them more stable and less reactive with feed ingredients in the digestive tract (Chae, S. P. and Acda, 2002). Nonetheless, its expensive cost restricts its use,

particularly when significant amounts of feed are required for fattening or reproduction purposes. Thus, a useful tactic could be to partially substitute it with less expensive inorganic sources, while still maintaining adequate Cu availability for the animal. Another source of inorganic Cu is monovalent Cu oxide (Cu_2O) which has not been previously authorized by the European Union (EFSA, 2016). Unlike $CuSO_4$, Cu_2O is a water non-soluble compound that has the highest Cu concentration in the market. Due to the high Cu% in Cu_2O, smaller quantities of them are used in broiler chicks' feed compared to this of $CuSO_4.5H_2O$ (Baker et al., 1991). A relatively recent experiment (Hamdi et al., 2018) demonstrated improved growth performance and lower accumulation of Cu in the liver of broiler chickens with therapeutic concentrations of Cu_2O compared with animals fed $CuSO_4$. However, there is limited knowledge about the impact of this recently authorized Cu source on the performance of poultry and swine, particularly with respect to its effects on gut microbiota.

6. Growth promotion

One of the primary objectives when evaluating in-feed additives is to assess their potential to enhance performance. Producers aim to improve performance, making it a crucial metric for measuring the efficacy of any feed additive. Studies conducted in the past have shown that providing dietary Cu to poultry and swine beyond the minimum requirement levels established by the NRC can result in improved performance (Hill et al., 2000; Skřivan et al., 2000) and its growth-promoting effects have been credited to its bacteriostatic and bactericidal properties (Stahly et al., 1980). Indeed, adding 150 to 250 mg/kg Cu to the diet of broiler chickens has been found to enhance their growth through the regulation of microflora in the intestine (Arias and Koutsos, 2006). However,

a study conducted by Zhou et al. (1994) demonstrated that administering Cu intravenously to weaned pigs, at a dosage of 200-250 mg Cu/kg in feed, could promote their growth. This finding further supports the idea that Cu's effects on growth are not solely due to its antimicrobial properties in the GIT, but also involve systemic mechanisms. Even so, it cannot be completely ruled out that there may be an indirect antimicrobial effect since excessive Cu given intravenously may excrete into the digestive tract, potentially affecting the microbiota.

Supplementation of high doses of Cu (100-250 mg/kg) to weanling pigs' diet has been shown to enhance their average daily gain (ADG) and average daily feed intake (ADFI), as reported in several studies (Cromwell et al., 1998; Hill et al., 2000; Pérez et al., 2011). The greater ADFI reported for pigs fed Cu-supplemented diets may be attributed to Cu's ability to increase the mRNA expression of neuropeptide Y (Li et al., 2008) which is known to stimulate feed intake (Gehlert, 1999). The circadian expression of feed intake in animals may be influenced by various signals, including growth hormone-releasing hormone (GHRH) and somatostatin, as well as neuropeptide Y (NPY), noradrenaline, galanin, and orexin, among others (Yang et al., 2011). Accordingly, Cu could play a role in regulating neuroendocrine activity in the brain since it can cross the blood-brain barrier in the form of a free ion. This could potentially affect the levels of neuropeptides associated with Cu (Choi and Zheng, 2009). Studies have shown elevated dietary Cu levels (175 and 250 mg/kg) seem 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) alongside the reduction on mRNA abundances of anorexigenic proopiomelanocortin in the hypothalamus (Zhu et al., 2011). An alternative metabolic pathway involves an increase in growth hormone mRNA expression in the pituitaries,

which is triggered by an elevation in GHRH mRNA levels in response to 125 mg/kg of Cu (Yang et al., 2011). In another study, it was found that the addition of 150 mg Cu/kg resulted in an increase in the mRNA expression of ghrelin (Gonzalez-Esquerra et al., 2019) which is commonly referred to as the "hunger hormone" due to its role in regulating feed intake and body weight in pigs.

Another proposed mechanism for the improvement of growth performance by Cu involves the stimulation of enzymes engaged in nutrient digestion (Dove, 1995). In fact, the addition of high concentrations of Cu has been shown to increase the activities of lipase and phospholipase A in the small intestine (Luo and Dove, 1996). This effect may lead to greater absorption of fatty acids and subsequently improve growth performance. However, supplemented diets for growing pigs with 150 mg/kg of Cu did not make any improvements in apparent total tract digestibility of energy or true total tract digestibility of fat (Espinosa et al., 2019, 2017). The addition of 150 mg/kg of Cu to diets for growing pigs resulted in increased levels of lipoprotein lipase and FABP1 in the subcutaneous adipose tissue and liver, respectively (Espinosa et al., 2020). As a result, the enhanced lipid metabolism and subsequent improvement in energy utilization may have led to the observed improvement in the pigs' growth performance when they were fed the Cu-supplemented diets (Espinosa et al., 2020).

Adding Cu to the diets of weanling pigs led to a decrease in the concentration of tumor necrosis factor α and an increase in the activity of superoxide dismutase in their blood serum (Espinosa et al., 2019; Gonzales-Eguia et al., 2009). This suggests that the improved growth performance seen in pigs fed the Cu-supplemented diets may have been due to an enhanced antioxidant capacity and humoral immune response, which could protect them from infections and diseases.

The presented studies demonstrate the potential of high dietary Cu supplementation to enhance the growth performance of swine and poultry. However, understanding the mechanism of action of Cu requires a broader consideration of its various modes of action and parameters beyond its antimicrobial properties in the GIT. Therefore, it is crucial to consider factors such as Cu's systemic effects on gene abundance and immune response, which also appear to play a role in promoting growth.

7. Consequences of excess Cu in the environment

The proliferation of antibiotic resistance determinants (i.e., antibiotic resistance genes; ARGs) in the environment has become a growing concern due to the excessive use of antibiotics (Wright and Poinar, 2012). This has led to the recognition of ARGs as an environmental pollutant and a significant public health issue (Stoll et al., 2012; Xiong et al., 2014). However, it has been shown that the development and dissemination of ARGs cannot be attributed solely to antibiotics, as there are multiple contributing factors (Andersson and Hughes, 2010). Animal feed supplemented with metals can also exert selective pressure for the long-term development of ARGs (Baker-Austin et al., 2006; Song et al., 2017). A direct correlation between the presence of heavy metals such as Cu and ARGs has recently been established. The co-selection of ARGs due to heavy metal exposure has been identified as a significant contributor to the rise in ARGs abundance, serving as a selective factor in their proliferation (Allen et al., 2010; Stepanauskas et al., 2006). A variety of mechanisms have been identified for the co-selection of metal resistance genes (MRGs) and ARGs in the environment (Ji et al., 2012). When considering the issue of environmental metal stress acting as a co-selection agent to facilitate the

proliferation of ARGs, it is worth noting that ARGs and MRGs share common modes of action, which contributes to the pollution of the environment with ARGs (Li et al., 2017).

This review has consistently noted the poultry and swine industry's practice of supplementing diets with pharmacological doses of Cu for many years. This supplement not only affects the availability of other nutrients within the animal's digestive tract (reviewed in the previous paragraphs) but also results in a significant quantity of undigested elements in the manure. Indeed, supplementing young pigs' diets with 250 mg/kg of Cu resulted in a twelve-fold increase in fecal Cu excretion compared to a diet containing 10 mg/kg (Armstrong et al., 2004). Using this manure as a fertilizer results in entering human and livestock food chains through crops and contaminated water, posing a significant threat to the environment and public health (EFSA FEEDAP, 2016). Consequently, the use of this mineral as a growth promoter has emerged as a major concern.

7.1. Development of Cu resistance

Cells have developed various mechanisms to regulate the levels of free Cu in their compartments due to their crucial role as a cofactor for certain enzymes. However, excessive concentrations of Cu and other metals can lead to cellular damage, such as altered enzyme specificity and disrupted cellular functions (Bruins et al., 2000). Consequently, cells activate resistance mechanisms to counteract the toxic effects of Cu (Rensing and Grass, 2003; Waldron and Robinson, 2009).

Gram-positive and Gram-negative bacteria rely on Cu-transporting P-type ATPases located in the cytoplasmic membrane to provide protection against Cu (Hasman et al., 2005). While in Gram-negative bacteria, ATPases export Cu across the inner membrane

to the periplasmic space, in Gram-positive bacteria, they export Cu out of the cytoplasm across the cell membrane (Hodgkinson and Petris, 2012). Extensively researched prokaryotic Cu transporters are the *CopA* and *CopB*. Cu is primarily utilized in the periplasm of bacteria, where it contains several Cu-dependent enzymes. Additionally, bacteria have developed a second mechanism for Cu tolerance, using multicopper oxidases. Under aerobic conditions, multicopper oxidases oxidize Cu^{+} to the less toxic Cu^{2+}, thereby conferring Cu tolerance to the bacteria (Hodgkinson and Petris, 2012). All of these mechanisms are chromosomally encoded defenses against toxic Cu levels. In addition, Gram-negative bacteria may also possess plasmid-encoded Cu resistance determinants. These determinants (e.g., *pco*, *cop,* and *tcrB*) are often found in environments with exceptionally high levels of Cu (Hasman et al., 2005). Many studies have investigated the associated genes in several bacterial species isolated from food-producing animals (Table 5). Indeed, cultivation-dependent studies demonstrated that high doses of dietary Cu (125 to 208 mg/kg) could be linked to acquired Cu resistance in *Enterococcus faecium* and *E. faecalis* via the plasmid-borne Cu resistance gene *tcrB* (Amachawadi et al., 2011, 2010; Hasman et al., 2006). It is interesting to note that in more recent studies, there was no evidence found to support the idea that high Cu supplementation affects Cu resistance in *E. faecalis* isolates obtained from swine feces (Capps et al., 2020; Villagómez-Estrada et al., 2020).

Table 5. Examples of Cu-resistance genes detected in bacteria from livestock adapted from Rensing et al. (2018)

Cu- resistance gene	Bacterial species	Animal origin
pcoA, pcoD	*Salmonella enterica serovar Mbandaka*	Pigs, cattle, poultry
	S. enterica serovar Derby	
	S. enterica serovar Heidelberg	
	S. enterica serovar Typhimurium	
	S. enterica serovar Worthington	
	S. enterica serovar Rissen	
	S. enterica serovar Agona	
	S. enterica serovar Senftenberg	
	S. enterica serovar London	
	S. enterica serovar Ohio	
	Escherichia coli	
	Histophilius somni	
copB	*Staphylococcus aureus*	Pigs, cattle, poultry
	Staphylococcus epidermidis	
	Staphylococcus haemolyticus	
	Staphylococcus hominis	
	Staphylococcus lentus	
	Staphylococcus pasteuri	
	Staphylococcus rostri	
	Staphylococcus sciuri	
	E. coli	
cueO	*E. coli*	Poultry
cusC	*E. coli*	Poultry
mco	*S. aureus*	Pigs

7.2. Co-selection of antibiotic resistance

The emergence of antibiotic-resistant bacteria is compromising the efficacy of antimicrobial therapy, making antimicrobial resistance one of the most significant threats to global public health. For an extended period, metals such as Cu and Zn have been associated with the development of antibiotic resistance in environmental bacteria (Calomiris et al., 1984; Wales and Davies, 2015). Several studies have established a connection between the presence of either of these metals in environmental samples and the concurrent presence of antibiotic and metal-resistant populations (Becerra-Castro et al., 2015; Lo Giudice et al., 2013), or a higher incidence of antibiotic-resistant genes/organisms in environments contaminated with metals compared to those free of

metal contamination (Hu et al., 2016; Lin et al., 2016; Xu et al., 2017). A strong association between higher Cu levels and an increase in antibiotic resistance genes (ARGs) has been found in a study examining the impact of Cu contamination on agricultural soils (Hu et al., 2016). Furthermore, as the concentration of Cu increased, the frequency of mobile genetic elements (MGEs) also increased, suggesting that Cu-selected ARGs could potentially be transferred to other bacteria (Hu et al., 2016). Due to their non-degradable nature, heavy metals tend to accumulate faster in the environment than certain antibiotics, which may result in a stronger selective pressure than antibiotics themselves under certain circumstances (Song et al., 2017). Co-selection mechanisms can be classified into three categories and can occur either indirectly through co-resistance and cross-resistance mechanisms or directly through coregulation (as illustrated in Figure 4). Studies have shown that metals, including Cu, can impact the co-selection of antibiotic resistance (Berg et al., 2010; Hu et al., 2017, 2016; Zhao et al., 2019). However, it is still unclear whether metals select for general bacterial species that are more resistant to antibiotics or for specific resistant strains within species, which could pose a greater risk to the environment (Pal et al., 2017). Additionally, little is known about the role of metals in the co-selection of mobilized ARGs. While the dominant pathway for metal-induced co-selection of ARGs is unknown, evidence suggests that cross-resistance through efflux systems may be a likely mechanism (Flach et al., 2017; Pal et al., 2017).

Further research is required to gain a better understanding of the impact of metal-induced co-selection of ARGs in farm animals. Specifically, it is necessary to investigate the genetic context of co-located ARGs and MRGs in different environments, as well as their proximity to MGEs. Additionally, dose-response studies are needed to determine

the minimum co-selective concentrations for metals in various environments. The toxicity of metals is highly dependent on environmental conditions, and as a result, the degree of co-selection is likely to differ (Berendonk et al., 2015).

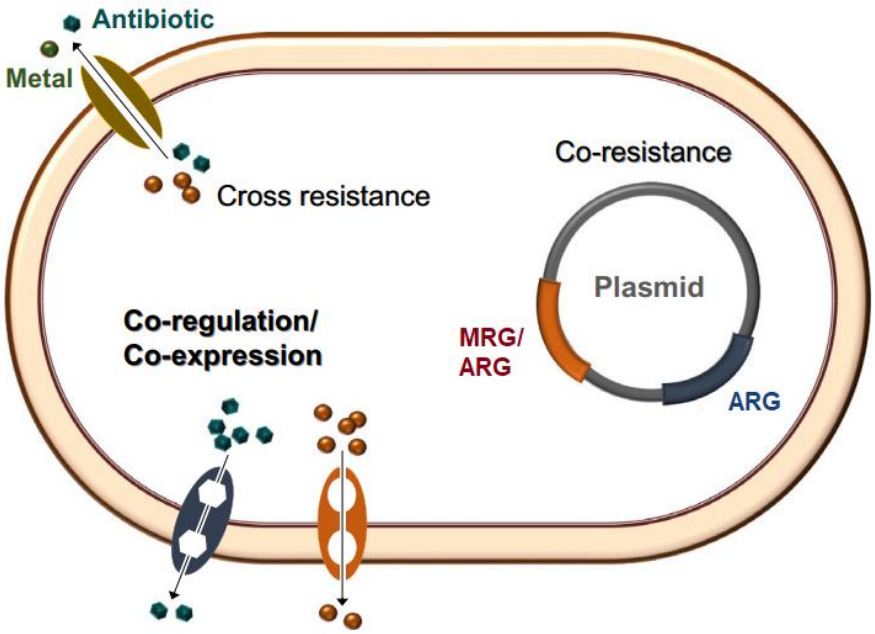

Figure 4. Overview of the mechanisms behind co-selection of antibiotic resistance adapted from Pal et al. (2017). ARG = Antibiotic resistance gene; MRG = metal

8. Strategies for sustainable usage

As previously discussed, Cu is an indispensable trace element that cannot be substituted. Historically, pharmacological or elevated doses of Cu have been successfully used in animal production to overcome critical stages in animals' lives, such as weaning, by promoting feed intake, controlling intestinal dysbiosis, enhancing innate immunity, and improving nutrient utilization. However, these high levels of Cu supplementation have negative effects on the environment and public health, necessitating a reduction in supplemented levels in food-producing animals. With various sources of Cu in commercial practice having different attributes, exploring strategies to supplement Cu

at reduced levels through more efficient and sustainable sources is a promising approach to optimizing Cu supplementation in the animal production industry. Moreover, managing animal housing and manure appropriately can reduce the amount of Cu and other trace minerals lost to the environment. The overall objective of sustainable Cu and other trace mineral use in animal production is to optimize animal health and productivity while minimizing the environmental impact of animal agriculture.

9. Perspective and hypotheses

After reviewing the existing literature and numerous conducted studies, we acknowledge the necessity and beneficial effects of Cu in agriculture and animal production. However, we must not overlook the potential harm that this trace element can cause to the environment. While extensive research has been done to evaluate Cu supplementation in farm animals, advancements in technology continuously offer new methods to explore the topic even further. For this thesis, we took advantage of relatively modern techniques such as 16S rRNA gene amplicon sequencing, gene abundance analysis, and high-throughput qPCR, in conjunction with traditional methods (e.g., analysis of blood parameters and bacterial isolation) to gain deeper insights into the function of Cu and the possibility of replacing the traditional source ($CuSO_4$) with a more efficient alternative (i.e. Cu_2O) for the benefit of animal health, farmers and the environment. Therefore, we designed three independent experiments to test the following hypotheses:

I. High concentrations of Cu result in improved growth performance, possibly due to alterations in the gut microbiota profile. However, an efficient Cu source such as Cu_2O in broiler diets is likely to have a more positive impact on growth performance than the conventional source ($CuSO_4$).

II. The improved growth performance observed with Cu is attributed to multiple modes of action and parameters, not just its antimicrobial properties. Moreover, the nature of Cu sources may affect their functionality in distinct ways.

III. Diets containing high levels of Cu co-select for antibiotic resistance in gut bacteria. Probably due to an increase in the prevalence of specific genes linked to Cu resistance in these bacteria.

IV. Replacing the standard water-soluble Cu source with a nonwater-soluble alternative may offer an alternative approach to mitigate the development of antimicrobial resistance. This, in turn, can lower the risk of environmental contamination without compromising productive performance.

OBJECTIVES

This Ph.D. dissertation is part of the SUMINAPP project (E! 11780), a project on the sustainable usage of trace minerals in animal production. This project aims to provide new insights on Cu and Zn supplementation in diets of food-producing animals for more sustainable practices. To achieve the main purpose, we designed three experiments, to shed light on the **function**, **mechanism of action,** and **environmental impact** of high-Cu diets in poultry and swine production through five specific objectives:

1. Explore the effect of high concentrations of Cu from two distinct sources ($CuSO_4$ and Cu_2O) on growth performance, intestinal microbiota profile, and antimicrobial resistance in broilers challenged with recycled necrotic enteritis litter (manuscript I).
2. Investigate the effect of high concentrations of Cu sources on oxidative status, inflammation, gene abundance, and cecal microbiome modulation in swine as potential mechanisms of action of high Cu (manuscript II).
3. Look into the fecal bacterial community composition, diversity, and the relative importance of different bacterial community assembly processes in swine fed high-Cu diets (manuscript III).
4. Determine the effect of high-Cu diets on the abundance of known genes that have been associated with Cu and other metal resistance in gut bacteria by means of a novel HT-qPCR metal resistance gene chip (manuscript III).
5. Explore the effect of high-Cu diets on the selection of phenotypic Cu resistance in specific populations of gut bacteria (*Escherichia coli*) (manuscript III).

MANUSCRIPT I

Effects of dicopper oxide and copper sulfate on growth performance and gut microbiota in broilers

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SUMMARY

An experiment was conducted to determine the effects of two sources of copper (Cu) from copper sulfate ($CuSO_4$) and dicopper oxide (Cu_2O, CoRouge) at three levels of inclusion (15, 75, and 150 mg/kg) on growth performance and gut microbiota of broilers. A total of 840 one-day-old male chickens (Ross 308) were weighed and randomly allocated to 7 dietary treatments: negative control (NC, a basal diet without Cu addition), and the NC supplemented with 15, 75, or 150 mg Cu/kg from $CuSO_4$ or Cu_2O (12 replicate pens/treatment, 10 chicks per pen). Broilers were challenged by reusing an old litter with high concentrations in *Clostridium perfringens* to promote necrotic enteritis. Broiler performance was registered at d 21, 35, and 42. Excreta samples were collected at d 14, 28, and 42 for antimicrobial resistance (AMR) analyses. At d 43, one broiler per pen was euthanized to obtain ileal content for microbial characterization. Body weight d 35 and daily gain d 42 improved ($P < 0.05$) in Cu_2O as Cu dose inclusion increased from 15 mg/kg to 150 mg/kg. Supplementation of 150 mg/kg of Cu from Cu_2O decreased the abundance ($P < 0.01$) of some families such as *Streptococcaceae* and *Corynebacteriaceae* and increased the abundance ($P < 0.05$) of some commensal bacteria like *Clostridiaceae* and *Peptostreptococcaceae*. Phenotypic AMR was not different among treatments on d 14 and 28. Isolated *Enterococcus* spp. from broilers fed the NC diet on d 42 showed higher ($P < 0.05$) resistance to enrofloxacin, gentamicin, and chloramphenicol compared with Cu treatments. By contrast, the isolated *Escherichia coli* from broilers fed 150 mg/kg of Cu, either from $CuSO_4$ or Cu_2O, showed higher ($P < 0.05$) resistance to streptomycin and chloramphenicol compared to the NC. This study suggests that supplementing 150 mg/kg of Cu from Cu_2O establishes changes in the gut microbiota by regulating the bacterial population in the ileum, which may explain the positive impact on broilers' growth performance.

1. Background

Copper (Cu) is an essential trace mineral in the poultry diet (Davis and Mertz, 1987). It is involved in immune function and oxidation, plays a significant role in iron metabolism (Kim et al., 2008; Ognik et al., 2016), and allows optimal growth performance by maintaining body functions (Banks et al., 2004b). The copper requirement for broilers is 5 to 8 mg/kg diet according to (NRC, 1994) and 4 to 10 mg/kg according to (FEDNA, 2018), but the maximum dosage authorized by (EFSA, 2012) in the European Union is 25 mg/kg. However, in many non-EU countries, therapeutic doses (125 to 250 mg/kg of Cu) of Cu from copper sulfate pentahydrate are being widely used as a growth promoter and antibacterial feed additive (Pang and Applegate, 2006).

Therapeutic doses of Cu may improve growth performance in animals by modulating the microbial population within the gastrointestinal tract (Arias and Koutsos, 2006) and, therefore, improving nutrient absorption (Bunch et al., 1965; Hawbaker et al., 1961). On the other hand, high Cu dosages influence antibiotic resistance development (Poole, 2017), and pollute the environment through higher Cu excretion (Malan et al., 2015). However, the antibacterial properties of Cu may depend on its redox state: Cu(I), the reduced cuprous form, has a stronger antibacterial effect in anaerobic conditions than Cu(II), the oxidized cupric form (Dunning et al., 1998). Besides, the different solubility and bioavailability of Cu sources may affect intestinal microbiota in a different way (Pang et al., 2009).

Copper sulfate ($CuSO_4$) is soluble in water (99%) and acidic solvents (Pang and Applegate, 2006) and has a Cu concentration of 25.4% (Baker, 1999). On the other side, dicopper oxide (Cu_2O, CoRouge, Animine) is a water non-soluble compound that has the highest Cu concentration in the market (75% of Cu). In a previous study, (Hamdi et al.,

2018) observed that therapeutic doses (150 mg/kg of Cu) of Cu_2O in broilers diet increased their body weight (BW), however, when 150 mg/kg of Cu from $CuSO_4$ was supplied growth performance was not modified, and feed efficiency reduced with 300 mg/kg addition. It was also suggested that excessive Cu accumulates in different organs, and free unbound copper in the blood may act as a strong oxidizing agent and cause a toxic response (Banks et al., 2004b; Reece, William O., Howard, H., Erickson, Jesse, Goff, P., Uemura, 2015). Nevertheless, there is no information about the effect of Cu_2O on gut microbiota.

Taking into account all the effects, we have hypothesized that using the most effective source (Cu_2O) could enhance performance at a therapeutic dose of 150 mg/kg, or even lower dosage, through changes in the gut microbiota. It was also hypothesized that differences between the sources may lead to a reduction in antimicrobial resistance (AMR) development caused by high Cu concentration.

Therefore, the objective of our study was to explore the effect of 75 or 150 mg/kg dose of Cu from Cu_2O on growth performance, intestinal microbiota profile, and AMR when it is compared to $CuSO_4$ in broilers challenged with recycled necrotic enteritis (NE) litter.

2. Materials and methods

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

2.1. Bird Management and Husbandry

The study was carried out at a commercial growing poultry unit (Tarragona, Spain). The room was provided with 84 solid-sided pens (0.8 × 1 m) in 4 lines of 21 pens divided by a central feeding aisle. A total of 840 one-day-old male chickens (Ross 308) were randomly allocated to one of 7 dietary treatments (12 replicate pens/treatment, 10 chicks per pen, and 0.64 m^2 per chick) according to initial BW and continuously controlled over 42 days. The average temperature was maintained at 35 ± 1 °C and was decreased gradually (at the rate of 3 °C per week) to 20 °C until d 42. The light cycle was provided 24 h/d from d 1 to d 2, 23 h/d from d 3 to d 10, and 18 h/day from d 11 to the last day of the experiment. Broilers were challenged by a recycled NE litter.

2.2. Necrotic Enteritis Challenge Procedure

The selection of the recycled litter material was made between four commercial poultry flocks based on signs of NE. The farm with the highest concentration of clinical NE and previously characterized for its content of mesophilic aerobic bacteria (> 10^5 CFU/g), *Enterobacteriaceae* (5.2 × 10^3 CFU/g), filamentous fungi and yeasts (2.2 × 10^3 CFU/g), and *Clostridium perfringens* (5.6 × 10^4 CFU/g) was selected. The floor area was covered with 10% clean wood shavings and 90% recycled litter material on the first day of the experiment. The challenging process comprised of exposing broilers to a contaminated litter characterized by high *Clostridium perfringens* counts was formerly used by (Abdelli et al., 2020).

2.3. Experimental Diets

A 3-phase feeding program was used, a starter phase from d 0 to d 21, a grower phase from d 22 to d 35, and a finisher phase from d 36 to d 42 (Table 6). Seven diets for each phase (21 diets in total) were prepared in a pelleted form (with a size of 1.8 mm for the starter phase and 3 mm for the grower and finisher). Dietary treatments were negative control (NC) diet without Cu supplementation and six additional diets in which 15, 75, or 150 mg/kg of Cu from $CuSO_4$ (Copper sulfate, 24.1% Cu, Manica Cobre S.L, Spain) or Cu_2O (CoRouge, 75.4% Cu, Animine, Sillingy, France) were added to the NC diet. The analyzed Cu concentration of each diet is presented in Table 7. The mineral-vitamin premix included in the diet was formulated and mixed without Cu. Diets were formulated to be isonutritive and to meet current estimates for nutrient requirements for growing broilers (FEDNA, 2018) and without antibiotics and growth promoters. Feed and water were offered *ad libitum*. Each diet was sampled in duplicate, grounded, and stored at 4ºC for their subsequent analysis.

2.4. Performance Measurements and Sample Collection

All the birds were weighed individually on d 0, 21, 35, and 42, and feed intake was recorded at d 21, 35, and 42. The average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated at the end of each phase and for the global period. Mortality and cause of death were also recorded. Excreta samples were collected at d 14, 28, and 42, from three animals per pen of all treatments (10 replicate pens/treatment), and a pool of excreta samples was made for each pen and day to analyze AMR. At d 43, 1 broiler per pen with a similar BW to the average of the

pen was selected. The broilers were stunned using an electrical stunner (Reference: 105523, FAF, France) and immediately exsanguinated to obtain ileal content.

Subsequently, based on the performance results, ileal content and excreta samples of NC and 150 mg/kg of Cu from $CuSO_4$ and Cu_2O treatments were used for the analysis of microbiota 16S rRNA gene, and AMR, respectively.

Table 6. Composition and nutrient content of the basal diet

Item	Starter	Grower	Finisher
Ingredients, %			
Ground corn	59.99	60.68	61.01
Soybean meal 47%	33.40	31.22	29.62
Soybean oil	2.55	4.27	5.66
Monocalcium phosphate	1.37	1.29	1.20
Calcium carbonate	1.28	1.21	1.21
Mineral-vitamin premix[1]	0.30	0.30	0.30
Sodium chloride	0.31	0.30	0.24
$_{DL}$-Methionine	0.25	0.25	0.24
$_{L}$-Lysine.HCl	0.15	0.10	0.12
$_{L}$-Threonine	0.05	0.05	0.06
Sodium bicarbonate	0.20	0.20	0.2
Choline chloride	0.15	0.13	0.14
Calculated composition, %			
ME Kcal/kg	2950	3050	3150
Crude Protein	21.20	20.00	18.50
Calcium	0.98	0.90	0.78
Phosphorus	0.68	0.65	0.62
Lysine	1.22	1.11	1.07
Methionine	0.56	0.54	0.52
Methionine + cysteine	0.91	0.87	0.84
Threonine	0.81	0.77	0.75
Tryptophan	0.23	0.23	0.21
Analyzed composition, %			
Dry matter	89.10	88.26	88.44
Crude Ash	5.69	5.48	5.08
Crude Protein	20.49	19.02	18.52
Crude Fat	5.43	6.73	7.76
Crude Fiber	3.04	2.96	3.43

[1]Provided per kg of diet: vitamin A (retinyl acetate), 17,000 IU; vitamin D_3 (Cholecalciferol), 3,500 IU; vitamin E (dl-α-tocopheryl acetate), 15 IU; vitamin K_3 (menadione sodium bisulfate), 2 mg; vitamin B_1, 1.6 mg; vitamin B_2, 4.16 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.012 mg; nicotinic acid, 21.2 mg; pantothenic acid (D-Ca pantothenate), 10.58 mg; biotin, 0.048 mg; folic acid, 0.8 mg; Zn (ZnO) 60.19 mg; Fe ($FeSO_4 \cdot 7H_2O$), 24 mg; Mn ($MnSO_4 \cdot H_2O$), 54.06 mg; I (KI), 0.6 mg; and Se ($NaSeO_3$), 0.18 mg; antioxidant, 0.8 mg.
ME = Metabolizable energy.

Table 7. Calculated and analyzed Cu concentration in the experimental diets

Item	Cu level, mg/kg			
	Calculated	Analyzed[1]		
		Starter	Grower	Finisher
Negative Control	7	6	9	6
$CuSO_4$	15	22	23	20
	75	78	103	88
	150	131	213	138
Cu_2O	15	30	23	20
	75	95	84	81
	150	139	152	169

[1]The values expressed as mean based on duplicate determinations.

2.5. Chemical Analysis

All the diets were analyzed according to standard methods for dry matter (*ISO 1999*), crude ash (*ISO 2002*), and crude protein (*ISO 1997*). Crude fat was analyzed with the Soxhlet method using Foss Soxtec/Hydrotec 8000™ System for total fat analysis, consisting of Soxtec™ 8000 extraction unit and Hydrotec™ hydrolysis unit, (FOSS Analytical, Denmark). The crude fiber content was also measured using the Weende method (NF V03–040). The copper content in all the diets was determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, model Optima 4300DV, PerkinElmer Inc.; Waltham, MA).

2.6. Microbiota 16S rRNA Gene Analysis

2.6.1. Library Preparation and Sequencing

Bacterial DNA was taken out from 250 mg of ileal content following the manufacturer's instructions with the commercial MagMAX CORE Nucleic Acid Purification Kit 500RXN (Thermo Fisher, Barcelona, Spain). Mock community DNA was involved as a control (Zymobiomics Microbial Community DNA). Samples were amplified using specific primers to the V3-V4 regions of the 16S rRNA DNA (V3-V4-Forward 5’-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', V3-V4-Reverse

5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3')

(Klindworth et al., 2013). The library preparation was performed in Microomics Systems SL (Barcelona, Spain).

2.6.2. Amplicon Sequences Processing and Analysis

Forward and reverse reads of raw demultiplexed were processed by following the methods and pipelines as implemented in QIIME2 version 2019.4 with defaulting parameters unless indicated (Bolyen et al., 2019). DADA2 was used for quality filtering, denoising, pair-end merging, and amplicon sequence variant calling (ASV, i.e., phylotypes) using *qiime dada2 denoise-paired* method (Callahan et al., 2016). Q20 was used as a quality threshold to define read sizes for trimming before merging (parameters: --p-trunc-len-f and --p-trunc- len-r). Reads were truncated at the place when the 75th percentile Phred score felt below Q20 for both forward and reverse reads. After quality filtering steps, the average sample size of reads was resolved and phylotypes were detected. ASVs were aligned using the *qiime alignment mafft method* (Katoh and Standley, 2013). The alignment was used to generate a tree and to calculate phylogenetic relations between ASVs using qiime phylogeny FastTree method (Price et al., 2010). To even sample sizes for the diversity analysis using *qiime diversity core-metrics-phylogenetic* pipeline, ASV tables were subsampled without replacement. The sample with the smallest size was discarded to take advantage of the sequencing depth of the dataset. Afterward, subsampling to the next lowest sample size was used for each comparison. Unweighted and weighted Unifrac distances were calculated to compare community structure (Lozupone et al., 2011). Taxonomic assignment of ASVs was

performed using a Bayesian Classifier trained with Silva V4 database (i.e., 99% OTUs database) using the *qiime feature-classifier classify-sklearn* method (Pedregosa et al., 2011). Unifrac distance matrices and ASV tables were used to calculate principal coordinates and construct ordination plots using the R software package version 3.6.0 (http://www.R-project.org).

2.7. Antimicrobial Resistance Analysis

Excreta samples (10 replicate pens/treatment) were analyzed for microbiological isolation of *Enterococcus* spp. and *Escherichia coli* (*E. coli*), using Slanetz-Bartley (Oxoid, UK) for 48 hours at 37 ºC and McConkey agar plates (Oxoid, UK) for 24 hours at 37ºC, respectively. Compatible colonies with *Enterococcus* spp. and *E. coli* were confirmed and identified by PCR (Dutka-Malen et al., 1995). Genotypic AMR analysis was done in all the bacterial isolates to detect the resistance genes for vancomycin (vanC1 and vanC2) (Dutka-Malen et al., 1995; Kariyama et al., 2000), tetracycline tet(M), and erythromycin erm(B) (Jacob et al., 2008). The detection of extended-spectrum beta-lactamases (ESBL) [blaSHV, blaCTX-M, blaCMY1, blaCMY2, and blaTEM] and carbapenemase-resistance (OXA-48) genes was performed as previously described by (Vidal et al., 2020). Also, copper (tcrB) and zinc (czcA) resistance genes were analyzed, as previously described by (Hasman et al., 2006).

In parallel, all *Enterococcus* spp. and *E. coli* isolates were tested for phenotypic antimicrobial sensitivity using the disk diffusion method, described by (Bauer, A.W.;Kirby, W.M.M.; Sherris, J.C.; Turck, 1966). Thirteen antimicrobial agents were used: penicillin G (10µg, Oxoid, UK) ampicillin (25 µg, BD, USA), imipenem (10 µg, BD, USA), vancomycin (30 µg, BD, USA), erythromycin (15 µg, BD, USA), tetracycline (30 µg, BD,

USA), ciprofloxacin (5 µg, BD, USA), enrofloxacin (5 µg, BD, USA), clindamycin (2 µg, BD, USA), gentamicin (10 µg, BD, USA), kanamycin (30 µg, BD, USA), streptomycin (10 µg, BD, USA) and chloramphenicol (30 µg, BD, USA). Cut-off values were those defined by the Clinical Laboratory and Standards Institute. Also, minimum inhibitory concentration tests were performed to assess the susceptibility of *Enterococcus* spp. and *E. coli* strains to copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) using the broth microdilution method as previously reported (Hasman et al., 2006).

2.8. Statistical Analysis

Growth performance data were analyzed as a complete randomized design with ANOVA using the GLM procedure of SAS software (SAS 9.4 Institute Inc., Cary, NC, USA). Homoscedasticity and variances normal distribution were checked before the analysis using the Shapiro-Wilk test and Levene's test from UNIVARIATE and GLM procedures, respectively. For growth performance parameters, the model included Cu source, Cu dose, and their interaction as a main effect and period as a random effect. The LSMeans statement was used to calculate mean values for each parameter. The AMR data were analyzed using the chi-squared test (Fisher Exact Test). For microbiota, Alpha and Beta diversity were analyzed using Vegan package and taxa differences with the MetagenomeSeq package in open source software RStudio v.3.5.1. Alpha diversity was calculated with raw counts based on Simpson, Shannon, and Inverse-Simpson estimators. Beta diversity was evaluated by multivariate ANOVA based on dissimilarities through envfit and adonis function. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log-normal mixture model, *P*-values were corrected by the false-discovery rate with metagenomeseq

package (Paulson et al., 2017). The experimental unit was the replicate, and statistical significance and tendencies were considered at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

3. Results

3.1. Growth Performance

Growth performance was lower than Ross 308 standards, which confirmed that the experimental challenge impaired the growth of the animals (Table 8). The mortality rate was 2.5% for the overall experiment, with no differences among the dietary treatments (results not presented). Broilers fed 150 mg/kg of Cu from Cu_2O had higher ($P = 0.033$) BW at d 35, and tended to have higher BW ($P = 0.053$) at d 42 than broilers fed 15 mg/kg of Cu from Cu_2O, a result which was not observed with $CuSO_4$. Broilers fed 150 mg/kg of Cu from Cu_2O had higher ADG ($P = 0.019$) than birds fed 15 mg/kg of Cu_2O, or NC at d 42. Supplementation of Cu from Cu_2O, irrespective of dose, tended to have lower ADFI ($P = 0.076$) and FCR ($P = 0.063$) than $CuSO_4$ supplementation at d 42.

Table 8. Growth performance (BW, ADFI, ADG, and FCR) of broilers fed dietary treatments[1]

Item		NC	$CuSO_4$, mg/kg			Cu_2O, mg/kg			SEM	P-value		
			15	75	150	15	75	150		Source	Dose	Source*Dose
BW, g	d 21	628.7	639.1	629.6	630.9	601.5	631.0	649.7	11.92	0.608	0.421	0.121
	d 35	1684.5^{ab}	1718.3^{ab}	1715.4^{ab}	1710.7^{ab}	1616.0^{b}	1718.2^{ab}	1783.6^{a}	29.21	0.748	0.038	0.033
	d 42	2420.8^{xy}	2505.6^{xy}	2489.7^{xy}	2473.8^{xy}	2404.4^{y}	2514.9^{xy}	2585.5^{x}	37.51	0.741	0.025	0.053
ADG, g/d	d 0-21	28.1	28.4	28.1	28.2	26.8	28.1	29.1	0.56	0.647	0.319	0.166
	d 21-35	75.0	77.0	77.6	76.7	72.5	77.7	80.6	1.70	0.912	0.060	0.113
	d 35-42	105.2	112.5	108.5	112	108.2	113.8	114.6	2.57	0.615	0.019	0.307
	d 0-42	56.6^{b}	58.6^{ab}	58.3^{ab}	57.9^{ab}	55.6^{b}	58.9^{ab}	60.5^{a}	0.88	0.932	0.013	0.019
ADFI, g/d	d 0-21	43.3^{AB}	44.0^{A}	43.0^{AB}	41.6^{B}	41.2^{B}	41.8^{AB}	43.3^{AB}	0.53	0.127	0.371	0.001
	d 21-35	133.1^{ab}	141.3^{a}	133.2^{ab}	137.2^{ab}	131.1^{b}	131.5^{b}	136.7^{ab}	2.03	0.032	0.062	0.043
	d 35-42	180.6	194.2	187.4	187.6	183.3	190.8	189	3.49	0.545	0.052	0.179
	d 0-42	96.1^{B}	101.5^{A}	97.4^{AB}	97.1^{AB}	94.8^{B}	96.5^{B}	98.7^{AB}	1.16	0.076	0.267	0.004
FCR, g/g	d 0-21	1.54	1.55	1.53	1.48	1.54	1.49	1.49	0.02	0.425	0.035	0.612
	d 21-35	1.78	1.84	1.73	1.80	1.81	1.70	1.70	0.04	0.142	0.027	0.594
	d 35-42	1.72	1.73	1.73	1.68	1.70	1.69	1.65	0.04	0.374	0.584	0.960
	d 0-42	1.70	1.73	1.67	1.68	1.71	1.64	1.63	0.02	0.063	0.004	0.663

[a-b] Means with different superscripts within a row indicate a significant difference of source*dose ($P \leq 0.05$).

[x-y] Means with different superscripts within a row indicate a tendency toward the significance of source*dose ($P \leq 0.1$).

[1]Data are means of 12 replicates per treatment.

NC = Negative control; SEM = Standard error of the mean; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.

3.2. Microbiota 16S rRNA Gene Analysis

Both alpha (Shannon, Simpson, and Inverse Simpson index) and beta diversity metrics were used to estimate microbial communities' diversity. Alpha diversity indices showed higher diversity and evenness ($P < 0.05$) in ileal microbiota of chickens fed 150 mg/kg of Cu from Cu_2O compared with NC and 150 mg/kg of Cu from $CuSO_4$ in Shannon index and the NC diet with all the indexes (Table 9). However, there were no differences in beta diversity among treatments ($P_{ENVFIT} = 0.4$, data not shown).

Table 9. Differences in α-diversity indices in ileal microbiota of broilers fed the NC diet or 150 mg/kg of Cu from CuSO4 and Cu2O at d 42[1]

Item	NC	$CuSO_4$, mg/kg	Cu_2O, mg/kg	SEM	P-value
		150	150		
Shannon	1.41^{B}	1.62^{B}	2.13^{A}	0.13	0.003
Simpson	0.57^{B}	0.65^{AB}	0.81^{A}	0.05	0.007
Invsimpson	3.01^{b}	3.30^{ab}	4.99^{a}	0.54	0.030

[a-b] Means with different superscripts within a row indicate significant differences ($P \leq 0.05$).

[1]Data are means of 12 replicates per treatment.

NC = Negative control; SEM = Standard error of the mean.

The relative abundance of phyla, families, and genera detected among the experimental groups are illustrated in Figure 5. Among 25 recognized phyla, Firmicutes was the major phyla (average 85.47%), followed by Cyanobacteria and Actinobacteria (average 5.81% and 5.49%, respectively). At the family level, out of 222 different families, 92% of the operational taxonomic unit (OTU) was allocated to 9 families of *Lactobacillaceae* (30 to 53%), *Streptococcaceae* (8 to 26%), *Enterococcaceae* (4 to 15%), *Clostridiaceae* (2 to 10%), *Peptostreptococcaceae* (3 to 10%), *Rivulariaceae* (4 to 7%), *Turicibacteraceae* (1 to 6%), *Corynebacteriaceae* (2 to 5%), and *Enterobacteriaceae* (0.2 to 1.7%), respectively. At the genus level, 85.88% of the OTU was assigned to 7 genera

of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Calothrix*, *Turicibacter*, and *Alkaliphilus*.

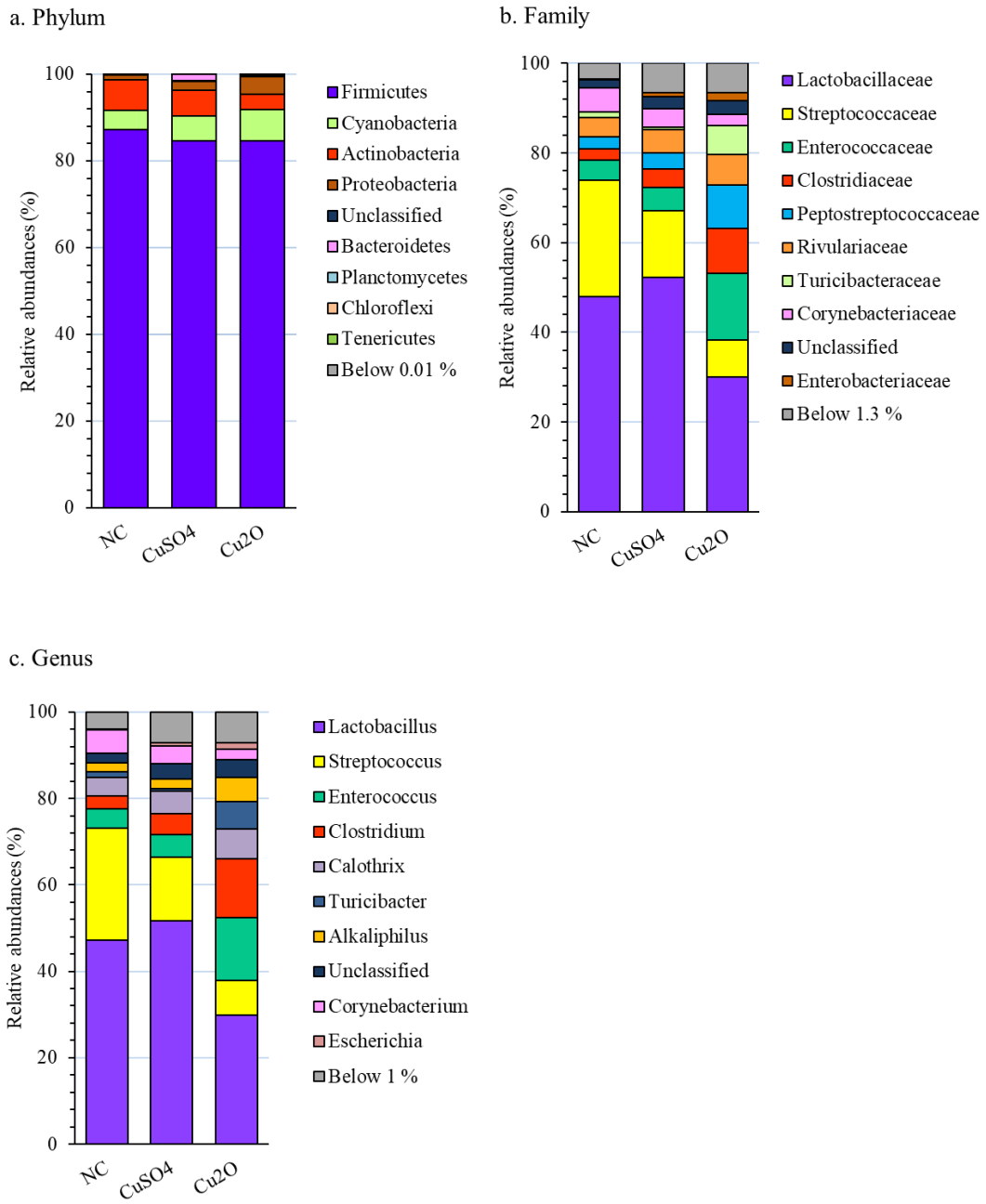


Figure 5. Relative abundance (%) of Top 10: phyla (a); families (b); and genera (c), in the ileum of different experimental groups. The rest of the taxonomic groups are pooled together (those representing less than a mean of 0.01, 1.3, and 1% of phyla, families, and genera, respectively). NC = Negative control; $CuSO_4$ = 150 mg/kg of Cu from $CuSO_4$; Cu_2O = 150 mg/kg of Cu from Cu_2O.

A more in-depth examination of the individual metagenomics profile changes was detected on the dietary treatments using log_2 changes. Broilers fed 150 mg/kg of Cu from $CuSO_4$ levels compared with those fed the NC (Figure 6) had significant differences in the relative abundance of Firmicutes (0.28 fold decrease; $P < 0.0001$) phyla, and some main families like *Lactobacillaceae* (0.29 fold decrease; $P < 0.0001$), *Streptococcaceae* (0.82 fold decrease; $P < 0.0001$), *Corynebacteriaceae* (0.52 fold decrease; $P < 0.0001$), *Enterococcaceae* (0.33 fold increase; $P < 0.0001$), *Peptostreptococcaceae* (0.37 fold increase; $P = 0.001$), *Clostridiaceae* (0.80 fold increase; $P = 0.004$), and *Enterobacteriaceae* (1.99 fold increase; $P = 0.013$).

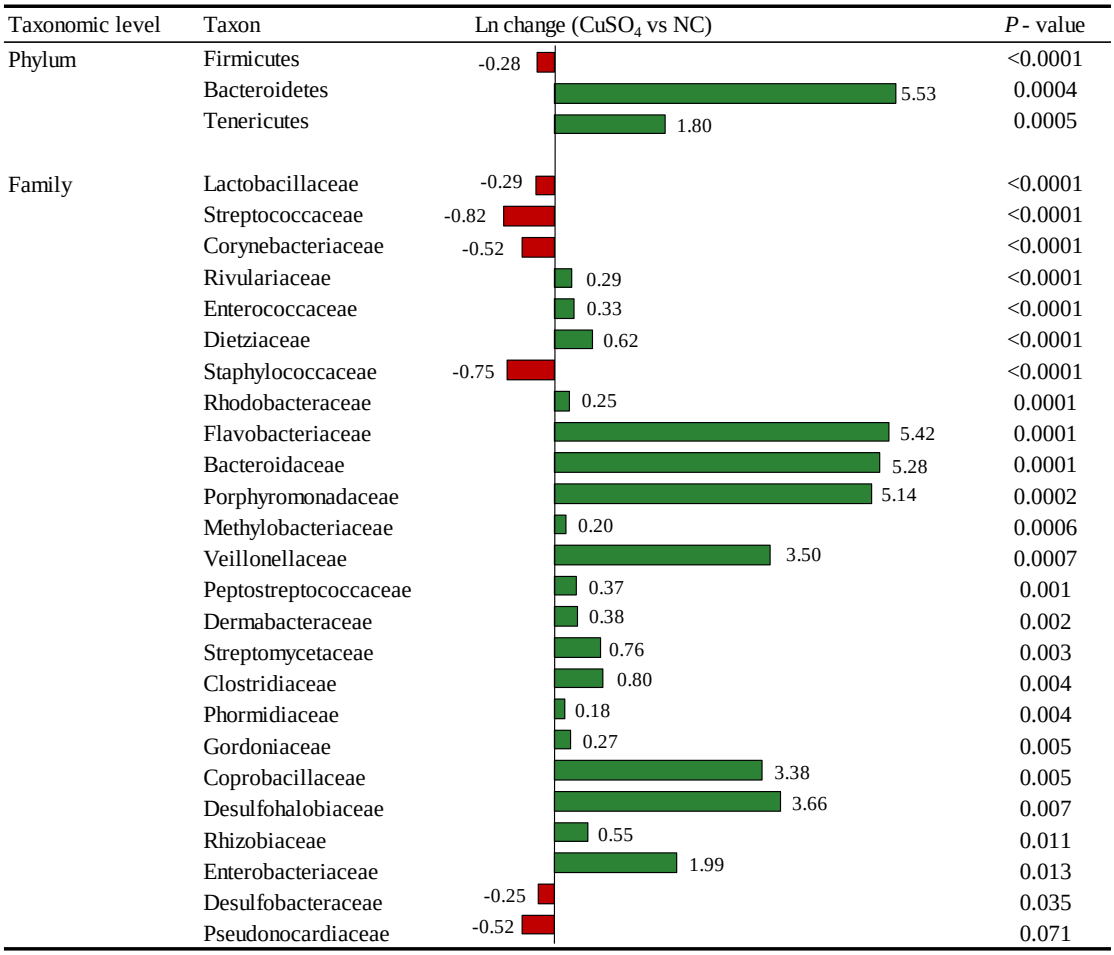


Figure 6. Differentially abundant taxa at the phylum and family level from the ileum on d 42 between 150 mg/kg of Cu from $CuSO_4$ and NC. Positive values (green color) and negative values (red color) indicate higher and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Data are means of 12 observations per treatment. NC = Negative control.

Broilers supplementation with 150 mg/kg of Cu from Cu_2O significantly changed the abundance of Firmicutes (0.43 fold decrease; $P < 0.0001$) phyla, and families of *Lactobacillaceae* (1.35 fold decrease; $P < 0.0001$), *Streptococcaceae* (2.02 fold decrease; $P < 0.0001$), *Corynebacteriaceae* (1.67 fold decrease; $P < 0.0001$), and *Enterobacteriaceae* (2.94 fold increase; $P = 0.0006$), compared with broilers fed the NC diet (Figure 7).

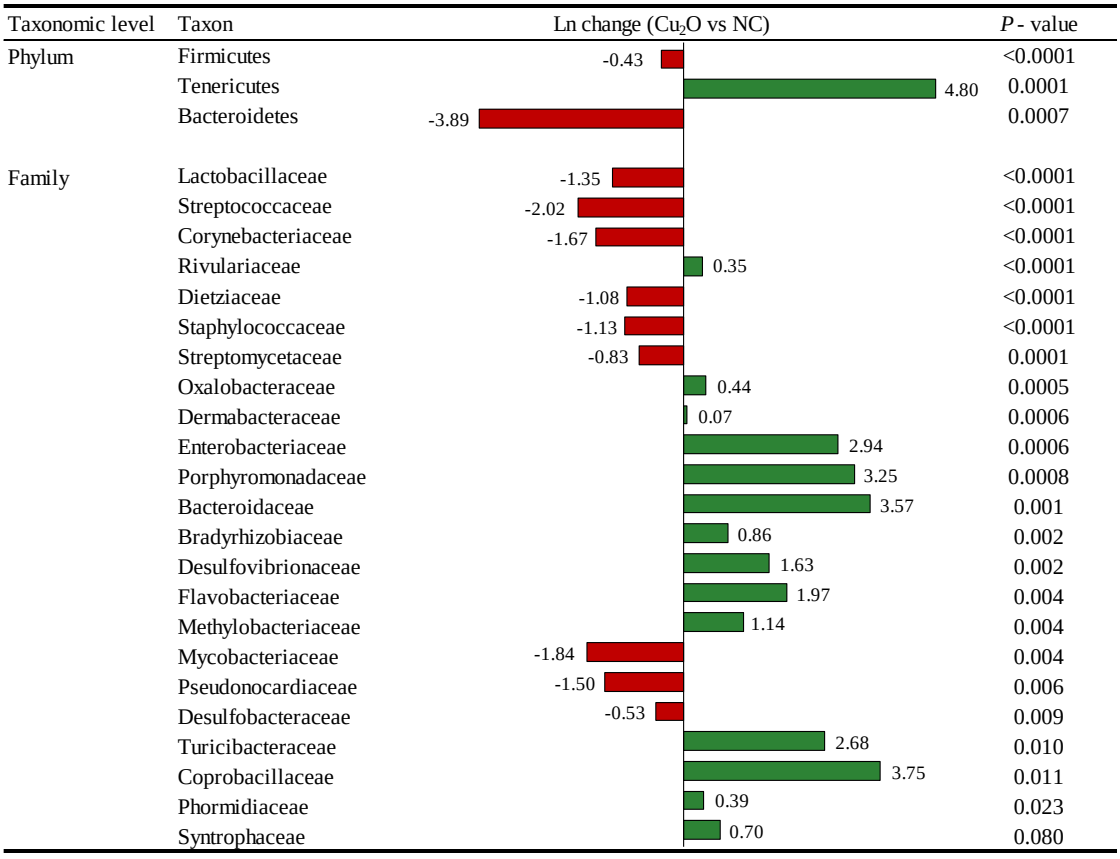


Figure 7. Differentially abundant taxa at the phylum and family level from the ileum on d 42 between 150 mg/kg of Cu from Cu_2O and NC. Positive values (green color) and negative values (red color) indicate higher and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Data are means of 12 observations per treatment. NC = Negative control.

The comparison between Cu sources revealed that the addition of Cu at 150 mg/kg from Cu_2O increased the abundance of *Peptostreptococcaceae* (1.37 fold; $P = 0.0004$), *Clostridiaceae* (1.11 fold; $P = 0.032$), and tended to increased the abundance of

Enterobacteriaceae (0.94 fold; *P* = 0.076), but reduced the amount of Firmicutes (0.15 fold; *P* < 0.0001) phyla, and *Streptococcaceae* (1.19 fold; *P* = 0.001) family compared with 150 mg/kg of Cu from $CuSO_4$ (Figure 8).

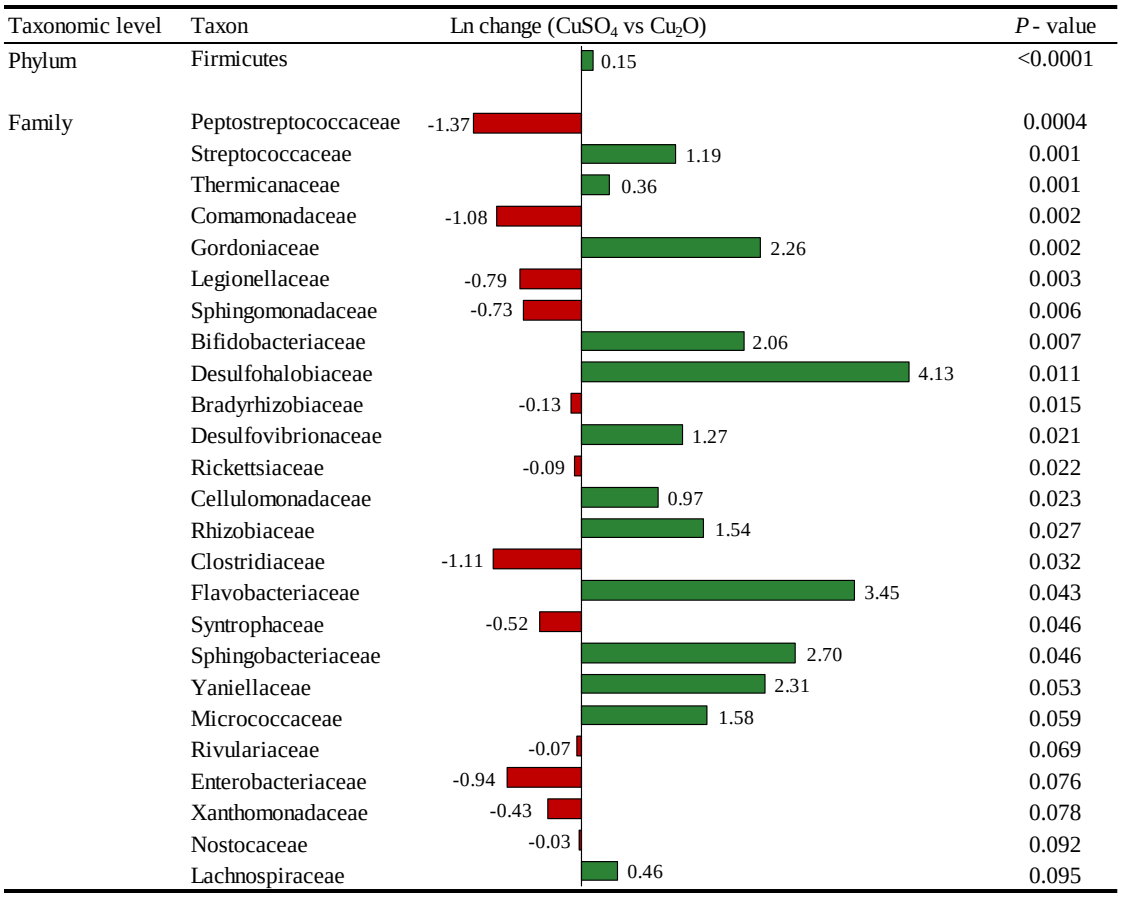


Figure 8. Differentially abundant taxa at the phylum and family level from the ileum on d 42 between 150 mg/kg of Cu from $CuSO_4$ and Cu_2O. Positive values (green color) and negative values (red color) indicate higher and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Data are means of 12 observations per treatment.

3.3. Antimicrobial Resistance Analysis

E. coli was isolated from more than 80% of the excreta samples and *Enterococcus* spp. from all samples. As regards to *Enterococcus* spp., *E. faecalis* was the most frequently isolated, representing 70% (in the $CuSO_4$ group) and 90% (in both NC and Cu_2O groups) of the total isolates at d 42. Interestingly, *E. faecalis* detection was

increasing according to days of the study (almost exclusively isolated from samples at d 42, and only detected in 2 samples from the $CuSO_4$ at d 14 and 28).

In the genotypical analysis, isolates were negative for ESBL and OXA-48 genes. The percentage of resistant strains of *Enterococcus* spp. was higher for the rest of the studied genes, in all the treatments, and days compared to *E. coli* strains (Table 10). Moreover, the frequency of tcrB resistant strains had an increasing trend over time in all treatments ($P > 0.1$). For the *E. coli* isolates, the rate of vancomycin-resistant strains was lower (< 20%) for vanC1 and vanC2 genes in all treatments and days. VanA and VanB genes were not detected in any isolate.

There were no differences among dietary treatments on the phenotypic AMR at d 14 and 28. However, for the isolated *Enterococcus* spp., broilers fed the NC diet had higher ($P < 0.05$) resistance to enrofloxacin, gentamicin, and chloramphenicol compared to animals fed 150 mg/kg of Cu from $CuSO_4$ and Cu_2O on d 42 (Figure 9.a). Conversely, the addition of 150 mg/kg of Cu from $CuSO_4$ and Cu_2O in the diet increased the *E. coli* resistance to streptomycin (78 and 56%, respectively) and chloramphenicol (56% on average) compared to the NC diet (11 and 0%, respectively; Figure 9.b). Remarkably high levels of AMR were observed in all *E. coli* strains, even in the NC group.

Table 10. Genotypical antimicrobial resistance in isolates of *Enterococcus* spp. and isolates of *E. coli* of broilers fed the NC diet and 150 mg/kg of Cu from $CuSO_4$ and Cu_2O at d 14, 28, and 42[1]

AMR genes[2]	Day 14			Day 28			Day 42		
	NC	$CuSO_4$, mg/kg	Cu_2O, mg/kg	NC	$CuSO_4$, mg/kg	Cu_2O, mg/kg	NC	$CuSO_4$, mg/kg	Cu_2O, mg/kg
Enterococcus spp.									
vanC1	70%	70%	100%	30%	20%	10%	100%	100%	70%
vanC2	60%	70%	100%	90%	90%	80%	100%	100%	100%
tetM	100%	90%	100%	100%	100%	100%	100%	100%	100%
ermB	100%	100%	100%	100%	100%	100%	100%	100%	100%
ESBL	0%	0%	0%	0%	0%	0%	0%	0%	0%
OXA-48	0%	0%	0%	0%	0%	0%	0%	0%	0%
tcrB	90%	80%	80%	80%	60%	90%	100%	100%	100%
czcA	10%	10%	0%	0%	10%	0%	50%	40%	0%
E. coli									
vanC1	20%	0%	0%	0%	20%	0%	0%	0%	0%
vanC2	20%	0%	30%	0%	10%	0%	0%	0%	0%
tetM	89%	10%	0%	89%	90%	86%	0%	0%	0%
ermB	78%	100%	10%	78%	80%	100%	50%	70%	20%
ESBL	0%	0%	0%	0%	0%	0%	0%	0%	0%
OXA-48	0%	0%	0%	0%	0%	0%	0%	0%	0%
tcrB	0%	0%	0%	0%	10%	0%	70%	20%	40%
czcA	100%	50%	50%	78%	100%	43%	0%	0%	0%

[1]Data are means of 10 replicates per treatment.

[2]Antimicrobial resistance genes: vancomycin (vanC1, and vanC2); tetracycline (tetM); erythromycin (ermB); penicillin, aminopenicillin and last generation cephalosporine (ESBL); imipenem (OXA-48); Cu (tcrB); Zinc (czcA).

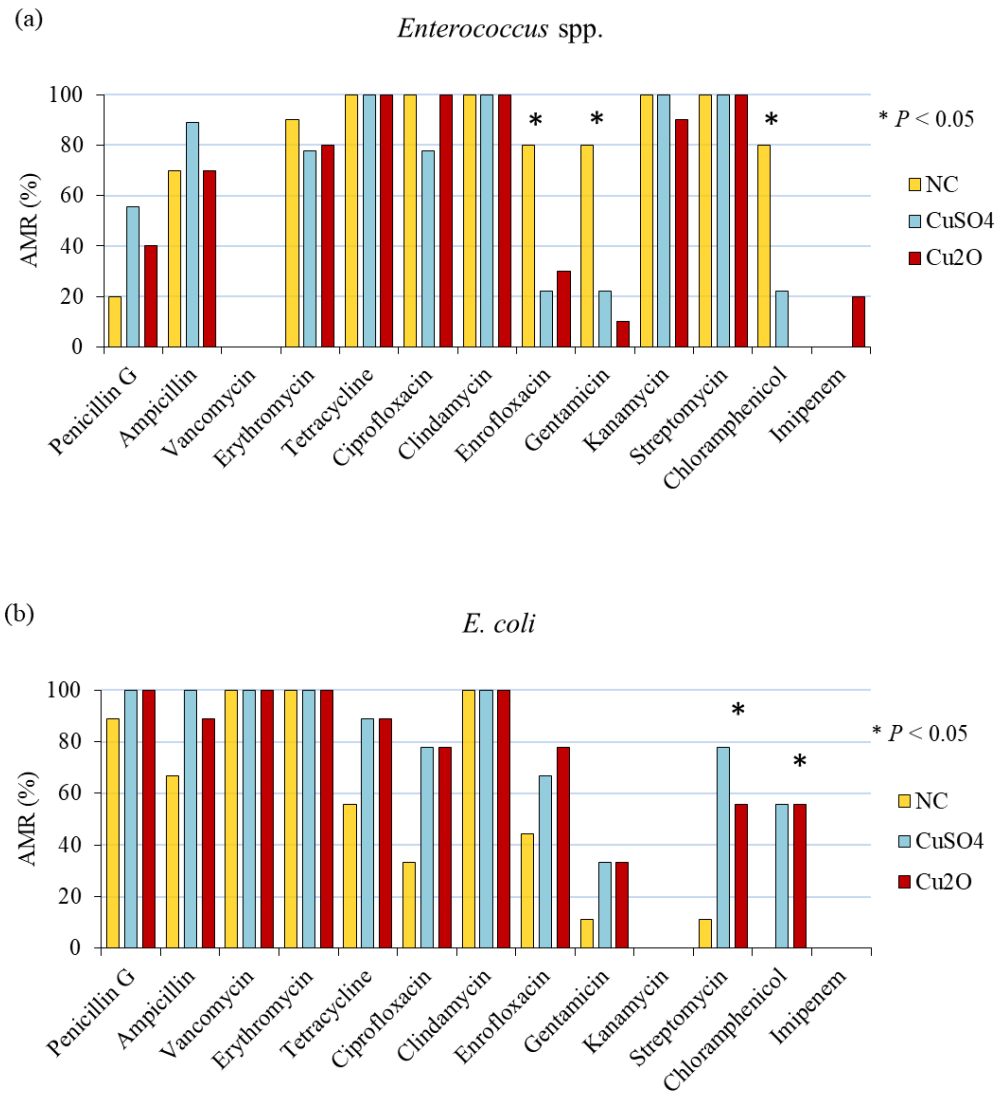


Figure 9. Phenotypic antimicrobial resistance in *Enterococcus spp.* isolates (a), and *E. coli* isolates (b) of broilers fed the NC diet and 150 mg/kg of Cu from $CuSO_4$ and Cu_2O at d 42. NC = Negative control; AMR = Antimicrobial resistance

4. Discussion

4.1. Copper Effects on Growth Performance

In this experiment, *Clostridium perfringens* challenge established by reusing 90% recycled commercial litter resulted in reduced growth performance in comparison with the standard Ross 308 values (17.8% decrease); this result is in line with (Abdelli et al., 2020) who observed a reduction of 21% by reusing commercial litter with NE. In this frame, our result revealed that Cu supplementation with Cu_2O at 150 mg/kg dose

increased the BW up to 10% at d 35 and numerically improved BW up to 7.5% at d 42 compared with 15 mg/kg dose. Whereas, supplied Cu as $CuSO_4$ has not modified growth performance at therapeutic doses (150 mg/kg). A numerical improvement of ADFI (up to 2%) and FCR (up to 1.8%) was also observed with Cu_2O supplementation in comparison to $CuSO_4$.

The hypotheses by which Cu stimulates growth include regulation of intestinal microflora (Pang et al., 2009), enhancement of neuropeptide Y and its mRNA expression level (Li et al., 2008), and improvement of dietary fat digestibility as a result of stimulated lipase and phospholipase activities (Luo and Dove, 1996).

Previous researches have described that dietary Cu can be beneficial for growth performance when fed over the minimum requirements in poultry and swine. In a study by (Arias and Koutsos, 2006) supplementing broilers' diet with 188 mg/kg Cu from $CuSO_4$ or tribasic copper chloride was also improved growth compared with those fed a non-supplemented diet, and growth improvement was the same with supplementation of sub-therapeutic antibiotics at d 45 under immune-challenging conditions (recycled vs. fresh litter). An 8.9% growth improvement and decreased FCR was observed by (Samanta et al., 2011) when broilers fed 150 mg/kg of $CuSO_4$ for 42 days. Similar positive effects of Cu on pigs were reported by (Villagómez-Estrada et al., 2020), where 160 mg/kg Cu from $CuSO_4$ or Cu hydroxychloride was able to increase performance at d 42.

The Cu response may, however, depend on the source. In an experiment conducted by (Hamdi et al., 2018), dietary level of 150 mg/kg of Cu from Cu_2O increased BW at d 35, whereas supplied Cu as $CuSO_4$ at the same dosage did not improve growth performance compared with 15 mg/kg. Our findings were in line with (Hamdi et al., 2018). In another study on broilers, (Lu et al., 2010) indicated that adding 200 mg/kg of

Cu from tribasic copper chloride improved ADG without increasing ADFI compared with 200 mg/kg of Cu from $CuSO_4$ or other doses of both sources.

The negligible impact of $CuSO_4$ on growth compared with other sources of Cu can be attributed to 1) damage to the mucosa and muscular layer in the intestinal tract (Chiou et al., 1999); 2) higher solubility (Pang and Applegate, 2006); 3) higher oxidation (Miles et al., 1998); 4) reduced phytase efficacy and decreased apparent phosphorus retention; and 5) toxicity (Banks et al., 2004a; Hamdi et al., 2018; Lu et al., 2010).

4.2. Copper Effects on the Gut Microbiota Profile

One of the growth-promoting actions of Cu has been credited to its antimicrobial effect in the gastrointestinal tract. Copper ions are toxic and can effectively kill bacteria or mold by denaturation or an oxidation mechanism (Kim et al., 2007; Lok et al., 2007). The electrostatic attraction combines ionic Cu with the plasma membrane and results in the cell membrane penetration through opening or closing of the membrane channel. This process leads to the leakage of intracellular ions and low molecular-weight metabolites by altering the permeability of cellular membranes (Tong et al., 2005). Meanwhile, Cu^{2+} enters into the cell, induces plasmid DNA degradation (Giannousi et al., 2014), and leads to bacterial death (Tong et al., 2005).

Some researchers have reported that high dietary Cu has affected gut microbiota profile and reduced the growth of pathogenic bacteria in animals (Højberg et al., 2005; Villagómez-Estrada et al., 2020; Zhang et al., 2017). (Xia et al., 2004) reported that the positive effect of Cu on weight gain in broiler chickens might be an outcome of the

significant reduction of the total pathogenic organism in the gut that intervenes with weight gain.

The analysis of ileal microbiota in the present study showed significant changes in some families of the gastrointestinal tract in broilers fed Cu. Supplementation of Cu ($CuSO_4$ or Cu_2O) in broilers' diet compared with the non-supplemented diet suppressed the abundance of *Streptococcaceae.* The genera *Streptococcus* is active in the process of simple sugar fermentation into lactate (Garvie, 1980; Zoetendal et al., 2012). Whereas, some species of the genus (e.g., *Streptococcus bovis*) are considered as major opportunistic pathogens, which can result in many diseases (Abdulamir et al., 2011; Munita et al., 2012; Qiao et al., 2014). A decreased *Streptococcus* abundance in colonic microbiota and increased growth performance have been observed in pigs fed 160 mg/kg of Cu (Villagómez-Estrada et al., 2020). Copper supplementation also declined the abundance of *Corynebacteriaceae*. Members of this family have been positively correlated with a wide range of severe infections, including opportunistic infections in both humans and animals (Prada et al., 1994; Zhi et al., 2017). Therefore, a lower proportion of *Corynebacteriaceae* may indicate a healthier intestinal environment.

Another family that responded to the treatments was *Enterobacteriaceae*, whose abundance was increased by Cu supplementation. The family *Enterobacteriaceae* includes 51 genera, which consist of commensal and pathogenic microorganisms (Janda, 2006). Opportunistic pathogens, e.g., *Citrobacter, Enterobacter, Escherichia, Klebsiella, Serratia,* and *Proteus* have been associated with diarrhea, urinary tract infections, mastitis, arthritis, and meningitis (Fairbrother et al., 2005; Nagy and Fekete, 2005). However, low levels (0.86 to 1.52%) of the mentioned pathogens were present in broilers fed Cu supplementation.

The reduction of families containing pathogenic bacteria as a result of high Cu supplementation may lay the ground for the growth of other families. Adding 150 mg/kg of Cu from $CuSO_4$ into the diet promoted the colonization of *Enterococcaceae*, *Peptostreptococcaceae*, and *Clostridiaceae* compared with the non-supplemented diet.

Enterococcaceae (*Enterococcus* spp.*)* belongs to the group of lactic acid bacteria. The genera consist of more than 20 species (Gomes et al., 2008). Some strains of this genus are capable of inhibiting the development of specific pathogens (Foulquié Moreno et al., 2006), and exhibit probiotic properties (Ó Cuív et al., 2013; Yadav and Jha, 2019). *Peptostreptococcaceae* and *Clostridiaceae* are members of Firmicutes phylum. *Peptostreptococcaceae* reported as normal commensal bacteria with a higher proportion in the gut microbiota of healthy animals than those experiencing dysbiosis of the intestinal microbiota. It indicates that this family helps preserve gut homeostasis (Fan et al., 2017). *Clostridiaceae* is one of the potential phylotypes involved in butyrate production from glucose, succinate, and lactate (Esquivel-Elizondo et al., 2017). Also, it has been highly correlated to protein and fat digestibility in dogs (Bermingham et al., 2017). The abundance of *Peptostreptococcaceae* and *Clostridiaceae* families have been previously shown to be associated with improved performance in broilers fed a mixture of organic acids with aromatic compounds or organic acids with medium-chain fatty acid plus aromatic compound (Abdelli et al., 2020).

On the other hand, the abundance of *Lactobacillaceae* was remarkably greater in broilers without Cu supplementation than those supplemented with Cu ($CuSO_4$ or Cu_2O). *Lactobacillaceae (Lactobacillus spp.)* is one of the main lactic acid-producing bacteria, and the primary end product of these bacteria is lactic acid (Garvie, 1980; Rajilić-Stojanović and de Vos, 2014) which has positive effects on growth. Interestingly, in a

study by (Gharib-Naseri et al., 2019), it has been asserted that increased *Lactobacillus* in the intestine may not indicate a healthier gut. Modified microbiota composition in challenging conditions could affect available nutrients for bacteria, and therefore, bacterial dynamics in the intestine (Stanley et al., 2012). Similar results were published by (Park and Kim, 2018). The authors reported an increase in the *Lactobacillus* population in the ileal digesta of broilers that received essential oils, but their BW were not significantly different from broilers in the non-supplemented group.

Comparing ileal microbiota of broilers fed Cu sources indicated that supplementation of Cu from Cu_2O was more effective than $CuSO_4$ towards the reduction of *Streptococcaceae* and development of *Peptostreptococcaceae* and *Clostridiaceae* which have beneficial properties. Bactericidal action of Cu contributed to the concentration of free ionic Cu in solution (Menkissoglu, O.; Lindow, 1991). Therefore, reduced copper states, such as Cu_2O, can provide Cu ion release more sustainably (Ren et al., 2011), and may exhibit a higher antibacterial activity (Dunning et al., 1998). Moreover, microbiota from the Cu_2O treatment group was more diverse, and OTU's were more evenly distributed, compared to the NC and $CuSO_4$ treatment groups. A correlation between FCR and richness and evenness indices has been previously observed by (Stanley et al., 2016). In their experiments, broilers with low FCR showed higher diversity than those with high FCR. Furthermore, in a recent study, (Villagómez-Estrada et al., 2020) discuss that reduction of opportunistic pathogens from one hand and development of saprophytic bacteria from the other hand, could lead to a significant improvement in intestinal nutrient absorption and, eventually, feed efficiency in pig fed 160 mg/kg of Cu from $CuSO_4$ or Cu hydroxychloride.

In agreement with (Villagómez-Estrada et al., 2020), our results suggest that adding 150 mg/kg of Cu, particularly Cu_2O, appears to improve intestinal microbiota profile and enhance chickens' performance by increasing the abundance of reportedly beneficial bacteria, such as *Peptostreptococcaceae* and *Clostridiaceae*, reducing the colonization of harmful bacteria, and increasing the diversity and evenness of ileal microbiota. However, further work is s required to understand how these changes in bacterial composition relate to metabolic changes in the host that ultimately lead to improved performance.

4.3. Copper Effects on the Antimicrobial Resistance

As an alternative to antibiotics, metal poisoning is used to destroy bacteria (Hao et al., 2016). This has resulted in the emergence and prevalence of AMR, representing a severe threat to public health worldwide (Hammerum and Heuer, 2009). High dietary Cu may have undesired effects, such as the growth of Cu-resistant bacteria (Pang et al., 2009). Copper resistance genes are usually located on plasmids and, in most cases, are transferable (Hasman and Aarestrup, 2002). These plasmids conferring resistance to copper (tcrB) have been identified in several *Enterococcus* species, including *E. faecium* and *E. faecalis*, in pigs, poultry, calves, and also humans (Torres et al., 2018). In *Enterococcus* spp. the most common genes conferring resistance to antibiotics are for erythromycin, tetracycline, and vancomycin (Oravcova et al., 2019; Tian et al., 2019).

On the other hand, there is high diversity and variants of *E. coli* strains integrating the normal gut microbiota and can cause severe diseases in both animals and humans, such as urinary tract infections, diarrhea, enteritis, and septicemia (Li et al., 2019). The routine use of antimicrobials in livestock for either 'prophylaxis' or 'metaphylaxis' has

represented a serious hazard for the selection of multidrug-resistant *Enterobacteriaceae* strains (Angulo et al., 2004). The effectiveness of treatments against *E. coli* is threatened by the dramatic increase of extended-spectrum beta-lactamases producing isolates worldwide (Carattoli et al., 2017; Livermore et al., 2007).

Regarding Cu resistance genes in the present study, a high prevalence of tcrB was detected in *Enterococcus* spp. isolates mainly on d 42, without any difference between groups. In the case of *E. coli*, tcrB was less frequent, but a higher prevalence was found at d 42. The presence of tcrB is not associated with Cu addition in the diet, as the presence of this gene was higher in the NC group. Another study has reported lower frequencies of tcrB gene in enterococcal isolates (34%) in broiler chickens, but higher levels of prevalence (76%) in pigs (Hasman and Aarestrup, 2002).

The presence of the zinc resistance gene czcA was barely detected in enterococcal isolates in the first two samplings (d 14 and 28). However, in the third sampling (d 42), the prevalence reached 50% in NC. Contrarily, in *E. coli*, the prevalence diminished significantly between samplings, going from 100% at d 14 to 0% at d 42. These differences were observed in all the treatments; therefore, they cannot be correlated with Cu addition in the diet.

Overall, a high prevalence of AMR genes was observed in *Enterococcus* spp. isolates. Erythromycin use is permitted in chickens, and laying hens and tetracyclines have been widely used as a growth promoter in animal husbandry (Granados-Chinchilla and Rodríguez, 2017). Therefore, the high prevalence of ermB and tetM could be explained by the frequent use of these antimicrobials in broiler farms over the years. A high prevalence of ermB and tetM in broiler excreta has been reported before in *Enterococcus* spp. isolates (Cauwerts et al., 2007; Hasan et al., 2018; Tremblay et al.,

2011). Also, (Cauwerts et al., 2007) found a correlation between the presence of ermB and phenotypical resistance to tetracyclines, mediated by several tet genes, tetM being among them.

The use of avoparcin, whose structure is similar to that of vancomycin, has decreased over the years in animal feed, and it has resulted in a reduction of the number of vancomycin-resistant isolates (Yazdankhah et al., 2014). Regarding the vancomycin resistance genes, high prevalences for the vanC1 and vanC2 genes were detected. The vanC genes are associated with low-level vancomycin resistance (de Moura et al., 2013; Watanabe et al., 2009), and are considered intrinsic in some enterococcal species, such as *E. gallinarum* and *E. casseliflavus* (Monticelli et al., 2018). The presence of these genes could be explained by the presence of this latter species on the excreta samples, and the possible transmission of plasmids containing the resistance genes between enterococcal species. Also, the presence of the vanC gene can explain the intermediate phenotypical resistance found in some of the analyzed isolates.

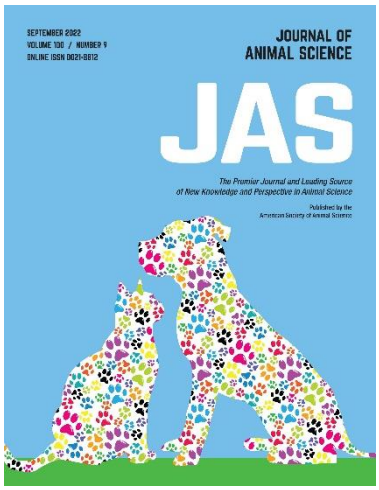
The phenotypical resistance in Enterococcal and *E. coli* isolates was high in almost all the antimicrobial agents. This high prevalence may be due to the fact that this study was carried in the north-east part of Spain, which has a very high density of pig production, where antimicrobial agents are widely used and the prevalence of AMR genes, principally in *E. coli* strains, has been highly reported (Vidal et al., 2020). Subsequently, observing any significant effect on interventional groups is challenging, given the high background levels of AMR in the non-supplemented group.

Likewise, most of the *E. coli* strains were resistant to vancomycin. This resistance was expected as vancomycin has been designed to kill a different type of microbe: gram-positive cocci. However, there were differences between Cu non-supplemented and

supplemented in *E. coli* isolates for streptomycin and chloramphenicol, where birds supplemented with Cu had higher resistance than non-supplemented. Although in (Agga et al., 2014) study with pigs, they did not observe any difference in the resistance prevalence of Streptomycin between Cu supplementations or without, but higher chloramphenicol prevalence was found in non-Cu-supplemented pigs. However, the mean prevalence was similar for both studies. For other antimicrobial agents (ampicillin, gentamicin, and kanamycin), the prevalences were similar between both studies, which suggests that Cu supplementation could have a similar effect in antimicrobial resistance for pigs and poultry. Controversially, in the *Enterococcus* isolates, no resistance was observed in vancomycin, and higher resistance prevalence was observed for non-Cu-supplemented birds in enrofloxacin, gentamicin, and chloramphenicol. Therefore, further studies with more controlled environmental conditions should implement in this field to assess the origin of these AMR.

5. Conclusion

To conclude, supplementation of the high dose of dicopper oxide (150 mg Cu/kg) was able to enhance the growth performance of broiler chickens raised under challenging conditions by modulating bacterial communities in the ileum. Finally, Cu addition did not alter the AMR genes in this study, which suggests that using broilers in a reused litter does not seem an appropriate method to check for AMR genes.



MANUSCRIPT II

How copper can impact pig growth: comparing the effect of copper sulfate and monovalent copper oxide on oxidative status, inflammation, gene abundance, and microbial modulation as potential mechanisms of action

Forouzandeh, A., Blavi, L., Pérez, J.F., D'Angelo, M., González-Solé, F., Monteiro, A., Stein, H.H., Solà, D., 2022. How copper can impact pig growth: comparing the effect of copper sulfate and monovalent copper oxide on oxidative status, inflammation, gene abundance, and microbial modulation as potential mechanisms of action. J. Anim. Sci.
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SUMMARY

The beneficial effect of elevated concentrations of copper (Cu) on growth performance of pigs has been already demonstrated, however, their mechanism of action is not fully discovered. The objective of the present experiment was to investigate the effects of including Cu from copper sulfate ($CuSO_4$) or monovalent copper oxide (Cu_2O) in the diet of growing pigs on oxidative stress, inflammation, gene abundance, and microbial modulation. We used 120 pigs with initial body weight (BW) of 11.5 ± 0.98 kg in 2 blocks of 60 pigs, 3 dietary treatments, 5 pigs per pen, and 4 replicate pens per treatment within each block for a total of 8 pens per treatment. Dietary treatments included the negative control (NC) diet containing 20 mg Cu/kg and 2 diets in which 250 mg Cu/kg from $CuSO_4$ or Cu_2O was added to the NC. On d 28, serum samples were collected from one pig per pen and this pig was then euthanized to obtain liver samples for the analysis of oxidative stress markers (Cu/Zn superoxide dismutase, glutathione peroxidase, and malondialdehyde, MDA). Serum samples were analyzed for cytokines. Jejunum tissue and colon content were collected and used for transcriptomic analyses, and microbial characterization, respectively. Results indicated that there were greater ($P < 0.05$) MDA levels in the liver of pigs fed the diet with 250 mg/kg $CuSO_4$ than in pigs fed the other diets. The serum concentration of tumor necrosis factor-alpha was greater ($P < 0.05$) in pigs fed diets containing $CuSO_4$ compared with pigs fed the NC diet or the diet with 250 mg Cu/kg from Cu_2O. Pigs fed diets containing $CuSO_4$ or Cu_2O had a greater ($P < 0.05$) abundance of genes related to the intestinal barrier function and nutrient transport, but a lower ($P < 0.05$) abundance of pro-inflammatory genes compared with pigs fed the NC diet. Supplementing diets with $CuSO_4$ or Cu_2O also increased ($P < 0.05$) the abundance of *Lachnospiraceae*, and *Peptostreptococcaceae* families and reduced ($P < 0.05$) the abundance of the *Rikenellaceae* family, *Campylobacter,* and *Streptococcus* genera in the colon of pigs. In conclusion, adding 250 mg/kg of Cu from $CuSO_4$ or Cu_2O regulate genes abundance in charge of the immune system and growth, and promote changes in the intestinal microbiota; however, Cu_2O induces less systemic oxidation and inflammation compared with $CuSO_4$.

1. Background

Copper (Cu) is part of many enzymes related to biological processes required for growth, development, and maintenance, such as cytochrome *c* oxidase, tyrosinase, *p*-hydroxyphenyl pyruvate hydrolase, dopamine beta-hydroxylase, lysyl oxidase, and copper-zinc superoxide dismutase (Cu, Zn-SOD; Gaetke and Chow, 2003). The beneficial effect of adding Cu in quantities that exceed the assumed requirement for maximizing the growth of pigs has been demonstrated (Cromwell et a., 1989; Hill et al., 2000), and the prevalence of diarrhea is also reduced if diets containing Cu above the requirement are fed (Pérez et al., 2011; Espinosa et al., 2017). These effects can be attributed to antimicrobial properties (Hill et al., 2000; Pang et al., 2009), and improved fat digestibility (Luo and Dove, 1996; Espinosa et al., 2021) of diets containing high Cu doses. However, excessive Cu may exert a toxic effect leading to cell death (Nawaz et al., 2006). Ozcelik et al. (2003) observed that excess Cu in the organism can cause cellular damage through the formation of free radicals, which induce oxidative stress (Gaetke and Chow, 2003), changes in lipid profile, and hepatic dysfunction (Sarkar et al., 2011).

Dietary Cu may be present in the oxidized cupric form (Cu^{2+}), or the reduced cuprous form (Cu^{+}; Linder and Hazegh-Azam, 1996). Although Cu can be provided in many different forms, Cu sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) is widely used in diets for pigs due to its relatively low cost and high solubility in water and acidic solvents (Pang and Applegate, 2006; Park and Kim, 2016). A new source of Cu, monovalent copper oxide (Cu_2O; Animine, Annecy, France), provided at up to 250 mg/kg diet results in improved growth performance and lower accumulation of Cu in the liver of broiler chickens (Hamdi et al., 2018; Forouzandeh et al., 2021) and weanling pigs (Bikker et al., 2017; Blavi et al., 2021) compared with animals fed Cu sulfate. However, no data are

demonstrating how Cu_2O impacts oxidative stress of pigs and limited information is available on the effects of Cu_2O on immune function, intestinal gene abundance, and the microbial population in the hindgut of growing pigs. To shed light on these issues, we used previously collected samples from phase one of a recently published study (Blavi et al., 2021). The previous study revealed that at the end of phase one therapeutic doses of Cu (either from Cu_2O or $CuSO_4$) were effective in improving pig growth but addition of Cu from Cu_2O reduced Cu accumulation in the liver and spleen compared with $CuSO_4$ addition. From this phase, pigs fed 250 mg Cu/kg from Cu_2O tended to have greater ADG compared with pigs fed 250 mg Cu/kg from $CuSO_4$.

Therefore, we hypothesized that dietary Cu_2O may result in less oxidative stress and inflammation than $CuSO_4$. It was also hypothesized that the addition of 250 mg/kg of Cu in diets for growing pigs modulates gene abundance and microbial population. Consequently, the objective of this experiment was to determine the effect of Cu_2O and $CuSO_4$ on oxidative stress, immune function, gene abundance, and gut microbiota of growing pigs.

2. Materials and methods

The Institutional Animal Care and Use Committee at the University of Illinois, USA, reviewed and approved the protocol for the animal part of the experiment. The experiment was a collaborative project between the University of Illinois, Urbana-Champaign, IL, USA, and Universitat Autònoma de Barcelona, Bellaterra, Spain. The animal part of the experiment was conducted at the University of Illinois whereas the sample analysis was conducted at Universitat Autònoma de Barcelona. Pigs used in the

experiment were the offspring of L 359 boars mated to Camborough females (Pig Improvement Company, Hendersonville, TN). Data for growth performance, carcass characteristics, bone mineralization, and organ accumulation of Cu, have been published (Blavi et al., 2021).

2.1. Animal management and husbandry

A total of 120 growing pigs (60 barrows, and 60 gilts) with average initial body weight (BW) of 11.5 ± 0.98 kg were allotted to a randomized complete block design with 2 blocks of 60 pigs with the weaning group being the blocking factor. There were 3 dietary treatments, 5 pigs per pen (half pens with 2 males and 3 females and the other half with 3 males and 2 females), and 4 replicate pens per treatment in each block. Thus, there were a total of 8 replicate pens per treatment in the experiment. Pigs were housed in pens with fully slatted floors and a dry feeder, and a nipple drinker was installed in each pen.

2.2. Diets and feeding

Three diets based on corn and soybean meal were formulated (Table 11). Diet contained 500 units of phytase per kilogram (Quantum Blue, AB Vista Feed Ingredients, Malborough, UK). Dietary treatments consisted of the negative control diet (NC) with 20 mg/kg of added Cu, and 2 diets in which 250 mg Cu/kg from either $CuSO_4$ or Cu_2O was added to the NC diet. Pigs were fed experimental diets for 28 d and had *ad libitum* access to feed and water throughout the experiment. Diets were prepared in a meal form and were formulated to meet current estimates for nutrient requirements for growing pigs

(NRC, 2012). The analyzed composition values of the experimental diets for DM, Ash, CP, AA, AEE, Ca, P, Cu and Zn are described in Blavi et al. (2021).

Table 11. Ingredient composition and nutrient content of the control diet in the experiment as fed-basis[1]

Item	Control diet
Ingredients, %	
Ground corn	59.75
Soybean meal, 48% CP	26.00
Dried whey	5.00
Fish meal	3.00
Soybean oil	3.00
Ground limestone	0.86
Dicalcium phosphate, 19% P	0.55
Lysine HCL, 78% Lys	0.35
DL-Met, 98% Met	0.09
Threonine, 98% Thr	0.10
Salt	0.40
Vitamin-mineral premix[2]	0.30
Phytase premix[3]	0.10
Titanium dioxide	0.50
Copper, mg/kg	20
Analyzed composition, %	
Dry matter	87.19
Ash	4.07
Crude Protein	17.88
Lys	1.38
Ether extract	5.5

[1]Two additional diets were formulated by adding 250 mg/kg of Cu from copper sulfate pentahydrate (25% Cu) or 250 mg/kg of Cu from copper (Ⅰ) oxide (75% Cu) to the control diet. The two copper sources were added at the expense of ground corn.

[2] Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D_3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 10 mg as copper sulfate and 10 mg copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.15 mg as sodium selenite and 0.15 mg selenium yeast; and Zn, 125.1 mg as zinc sulfate.

[3]Phytase premix was prepared by mixing 900 g ground corn and 100 g Quantum Blue 5000 G (AB Vista Feed Ingredients, Marlborough, UK) to provide 500 phytase units per kilogram complete diet.

2.3. Sample collection

On the last day of the experiment, a blood sample was collected from the jugular vein via vena-puncture of the pig in each pen that had a BW closest to the pen average

(4 barrows and 4 gilts per treatment). Samples were collected in 2 ethylenediaminetetraacetic acid (EDTA) vacutainers (BD Diagnostics, Franklin Lakes, NJ). To recover serum, vacutainers were centrifuged at 700 × g at 4ºC for 13 min. One set of serum samples was stored at -20ºC until analyzed for cytokines and chemokines. The other set of serum samples was stored at -80 ºC until analyzed for malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and Cu/Zn superoxide dismutase (SOD) activity.

Pigs from which blood samples were collected, were sacrificed to obtain liver, jejunum, and colon digesta. Liver samples were collected and immediately placed in liquid nitrogen and stored at −80 ºC for analysis of GSH-Px activity and MDA. Jejunum tissue was collected from the mid-jejunum (30 cm of the middle of the jejunum) by cutting pieces of 3 to 4 cm and removing the digesta. Jejunum tissues were thoroughly washed in ice-cold phosphate-buffered saline solution and placed into 2 mL RNAse-free vials to analyze gene abundance. Jejunum samples were frozen and stored at -80ºC immediately after collection. Colon content was also collected from the spiral colon approximately 50 cm from the cecum. The colonic digesta were analyzed for microbiota.

2.4. Oxidative stress marker and antioxidant analysis

Serum MDA was measured with a thiobarbituric acid reactive substances assay kit (Cayman Chemical, USA). Activities of SOD and GSH-Px in serum were determined by spectrometry following instructions of Ransod and Ransel kits, respectively (Randox, County Antrim, UK). All the samples were analyzed in duplicate. One gram of liver was mixed with 4 mL of sucrose buffer (0.32M) and homogenized with a tissue homogenizer and was immediately placed on ice. The content was then added to a centrifuge tube

and centrifuged at 3,000 × g at 4ºC for 15 min. The supernatant was filtered with a double paper filter and used to assess GSH-Px activity and MDA with the same method as used for serum samples.

2.5. Multiplex immunoassay

The following cytokines and chemokines were analyzed in serum: interferon alfa (INF-α), interferon-gamma (IFN-γ), interleukin 10 (IL-10), interleukin 1 beta (IL-1β), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor-alpha (TNF-α), interleukin (IL-12) using the 9-Plex Porcine ProcartaPlex™ Panel 1 (ThermoFisher Scientific, EXP090-60829-901). All samples were analyzed as recommended by the manufacturer and were read on a Luminex® 200™ (Luminex Co., TX, USA).

2.6. Gene abundance analysis

Jejunum tissues were analyzed to determine the abundance of 56 intestinal genes classified into 5 groups related to barrier function (BF), immune response (IR), nutrient transport (NT), gut enzyme/hormone (EH), and stress (ST) using an Open Array Real-Time PCR Platform (Applied Biosystems, Waltham, MA, US) as explained by González-Solé et al. (2020).

2.7. Microbiota 16S rRNA gene analysis

2.7.1. Library Preparation and Sequencing

Bacterial DNA was recovered from 250 mg of colon digesta following the manufacturer's instructions with the commercial MagMAX CORE Nucleic Acid

Purification Kit 500RXN (Thermo Fisher, Barcelona, Spain). Mock community DNA was involved as a control (Zymobiomics Microbial Community DNA). Samples were amplified using specific primers to the V3-V4 regions of the 16S rRNA DNA (V3-V4-Forward 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', V3-V4-Reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). The library preparation was performed in Microomics Systems SL (Barcelona, Spain).

2.7.2. Amplicon sequences processing and analysis

Forward and reverse reads of raw demultiplexed samples were processed following the methods and pipelines in QIIME2 version 2019.4 with defaulting parameters unless indicated (Bolyen et al., 2019). DADA2 was used for quality filtering, denoising, pair-end merging, and amplicon sequence variant calling (ASV, i.e., phylotypes) using *qiime dada2 denoise-paired* method (Callahan et al., 2016). Q20 was used as a quality threshold to define read sizes for trimming before merging (parameters: --p-trunc-len-f and --p-trunc- len-r). Reads were truncated at the place when the 75th percentile Phred score felt below Q20 for both forward and reverse reads. After quality filtering steps, the average sample size of reads was resolved and phylotypes were detected. ASVs were aligned using the *qiime alignment mafft method* (Katoh and Standley, 2013). The alignment was used to generate a tree and to calculate phylogenetic relations between ASVs using qiime phylogeny FastTree method (Price et al., 2010). To even sample sizes for the diversity analysis using *qiime diversity core-metrics-phylogenetic* pipeline, ASV tables were subsampled without replacement. The sample with the smallest size was discarded to take advantage of the sequencing depth of the dataset. Afterward,

subsampling to the next lowest sample size was used for each comparison. Unweighted and weighted Unifrac distances were calculated to compare community structures (Lozupone et al., 2011). Taxonomic assignment of ASVs was performed using a Bayesian Classifier trained with Silva V4 database (i.e., 99% OTUs database) using the *qiime feature-classifier classify-sklearn* method (Pedregosa et al., 2011). Unifrac distance matrices and ASV tables were used to calculate principal coordinates and construct ordination plots using the R software package version 3.6.0 (http://www.R-project.org).

2.8. Calculations and statistical analyses

Normality of residuals was verified using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC) and outliers were identified using PROC ROBUSTREG of SAS and removed. Oxidative stress markers, MDA, and cytokines were analyzed as a randomized complete block design, using the PROC MIXED of SAS with a model that included treatment as the main effect and block as a random effect. Mean values were calculated using the LSMeans statement. The pen was the experimental unit for all analysis. An alpha value of 0.05 was used to assess significance among means and tendencies were considered at $0.05 \leq P < 0.10$.

Gene abundance data were analyzed using the ThermoFisher Cloud software 1.0 (Applied Biosystems, Waltham, MA, USA) applying the $2^{-\Delta\Delta Ct}$ method for relative quantification and using the sample with the lowest abundance as a calibrator. Some parameters were adjusted: maximum Crt allowed was 26, AMP score < 1.240, Cq confidence > 0.8, and maximum standard deviation allowed between duplicates were fixed at < 0.38. Relative quantification values were checked for normalization by a log10 transform, and all the statistical analysis was performed with R 3.4.3 software (R

Development Core Team 2013) and Bioconductor (Gentleman et al., 2004). One-way ANOVA and Benjamini-Hochberg false discovery rate (FDR Q-value) to control multiple *P*-values were calculated (Benjamini and Hochberg, 1995) giving an upper bound of the expected proportion of false significant tests, that is, false significant treatment differences in mean abundance levels between treatments. For microbiota, Alpha and Beta diversity were analyzed using the Vegan package and taxa differences with the MetagenomeSeq package in open-source software RStudio v.3.5.1. Alpha diversity was calculated with raw counts based on Simpson, Shannon, and Inverse-Simpson estimators. Beta diversity was evaluated by multivariate ANOVA based on dissimilarities through envfit and adonis function. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log-normal mixture model after normalization with Cumulative Sum Scaling. *P*-values were corrected by FDR with the metagenomeseq package (Paulson et al., 2017). Statistical differences among the treatments were identified at *P*-values under 0.05 for the ANOVA and Tukey's analysis, and Q-values under 0.1 for the FDR.

3. Results

3.1. Oxidative stress markers, and malondialdehyde concentrations

Malondialdehyde, SOD, and GSH-Px in the serum were not different among dietary treatments (Table 12). However, pigs fed 250 mg Cu/kg from $CuSO_4$ had greater ($P < 0.05$) MDA in the liver compared with pigs fed the NC diet or the diet containing 250 mg Cu/kg from Cu_2O. The GSH-Px concentration tended to be greater ($P < 0.10$) in the liver of pigs fed 250 mg Cu/kg from Cu_2O compared with pigs fed the other diets.

Table 12. Oxidative stress markers (SOD and GSH-Px) and Malondialdehyde concentration at the end of the experiment in the serum and liver of pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O[1]

	NC	$CuSO_4$, mg/kg	Cu_2O, mg/kg
Item		250	250
Serum			
MDA, µM	9.13 ± 1.06	9.47 ± 1.57	10.43 ± 2.53
SOD, U/L	0.22 ± 0.00	0.20 ± 0.02	0.24 ± 0.06
GSH-Px, U/L	3919 ± 197.98	3646 ± 350.07	3626 ± 502.34
Liver			
MDA, µM	38.19 ± 5.65^{b}	42.96 ± 3.20^{a}	37.38 ± 1.35^{b}
GSH-Px, U/mg prot	1.12 ± 0.07^{y}	1.13 ± 0.05^{y}	1.19 ± 0.08^{x}

[1]Data are means of 8 observations per treatment.

$^{a-b}$ Means with different subscripts within a row differ ($P < 0.05$)

$^{x-y}$ Means with different subscripts within a row differ ($P < 0.10$)

3.2. Cytokine concentration

Among the studied cytokines, IL-4, IL-6, and IL-10 were not detected because the values were below the standard curve. No differences were observed among treatments for IFN-α, IL-8, and IL-12, but serum concentration of IFN-γ tended to be lower in pigs fed 250 mg Cu/kg from $CuSO_4$ or Cu_2O compared with those fed the NC (Figure 10). Pigs fed 250 mg Cu/kg from $CuSO_4$ tended to have greater ($P < 0.10$) IL-1β and had greater ($P < 0.05$) TNF-α concentration than pigs fed the diet containing 250 mg Cu/kg from Cu_2O or the NC.

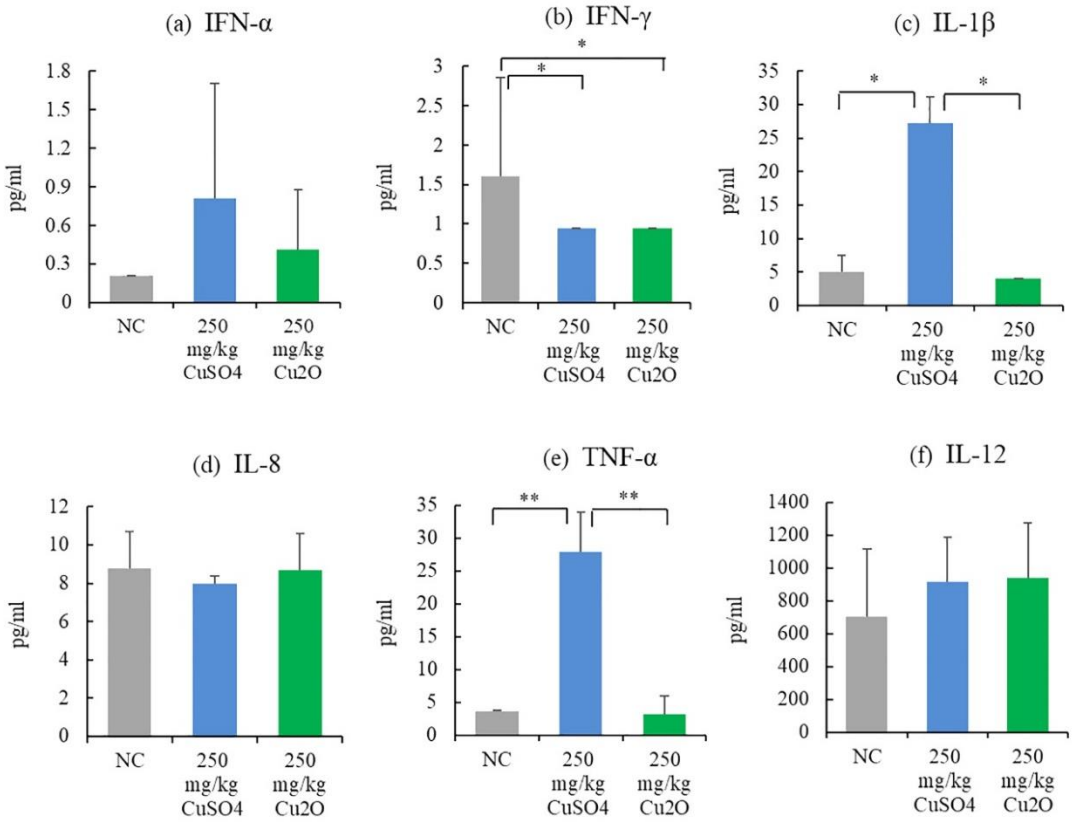

Figure 10. Cytokine concentration in the serum of pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O. Data are means of 8 observations per treatment. Error bars show the standard deviation. PROC MIXED significance, * indicates $P < 0.1$ and ** indicates $P < 0.05$.

3.3. Gene abundance

A heatmap was constructed to observe overall similarities among gene abundance profiles (Figure 11). A total of 47 genes were successfully amplified. Twenty-six genes involved in diverse physiological functions responded to the exposed diets ($P < 0.05$, $Q < 0.10$, Table 13). The *CLDN15*, *MUC2*, and *TFF3* genes from the BF group, *TLR2* gene from the IR group, *SLC39A4/ZIP4*, and *SLC5A1/SGLT1* from the NT group, *DAO1*, *IGF1R*, and *SOD2* gene from the EH group had a higher abundance in pigs fed 250 mg Cu/kg treatments compared with those fed the NC diet ($P < 0.05$). Pigs fed 250 mg Cu/kg from $CuSO_4$ had a lower ($P < 0.0.05$) abundance of *CCL20* (IR), and *SI* (EH) than pigs fed the NC diet, and a higher ($P < 0.05$) abundance of *SLC16A1/MCT1* gene (NT) than pigs fed the

NC diet or the diet with 250 mg Cu/kg from Cu_2O. However, pigs fed the diet containing 250 mg Cu/kg from Cu_2O had a lower abundance of *ZO1* (BF) compared with pigs fed NC or the diet containing 250 mg Cu/kg from $CuSO_4$, and these pigs also had a lower ($P < 0.05$) abundance of *HSPA4* and *IFNGR1* (IR), but an elevated ($P < 0.05$) abundance of *PYY* (EH) compared with pigs fed NC.

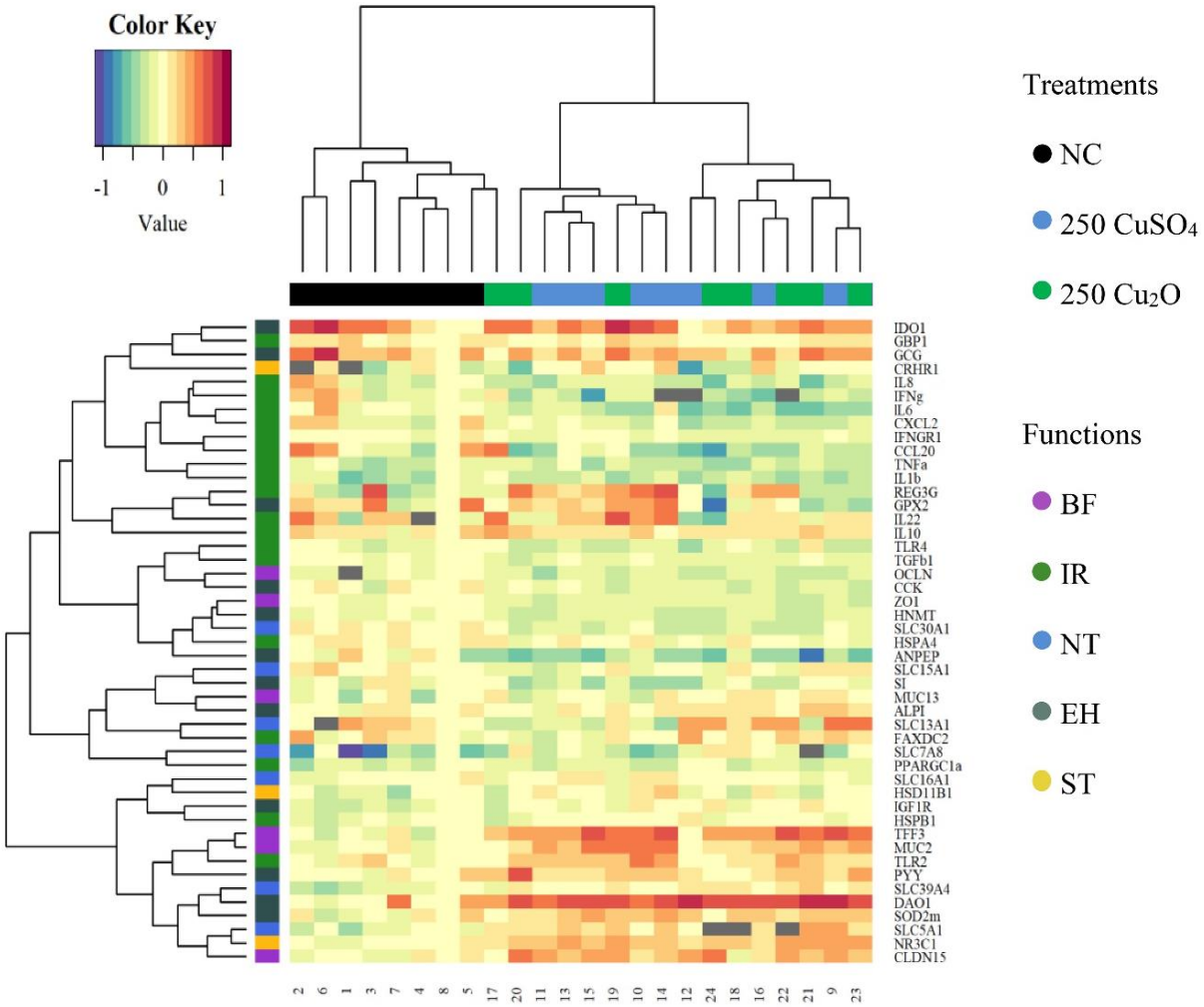

Figure 11. Heatmap representing the gene abundance of each sample in each gene. Each row is a gene, and each column is a sample. Samples are labeled with different colors representing every treatment (black for the negative control (NC), green for 250 mg Cu/kg from $CuSO_4$, and blue for 250 mg Cu/kg Cu_2O) and genes depending on its functional group (purple for barrier function-related genes (BR), dark green for immune response-related genes (IR), light blue for nutrient transport-related genes (NT), grey for enzyme/hormone-related genes (EH), and yellow for stress-related genes (ST)).

Table 13. Relative gene abundance differences of jejunum between pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O[1]

Function	Genes[2]	NC	$CuSO_4$, mg/kg	Cu_2O, mg/kg	Contrast Statistic[3]	*P*-value	*Q*-Value (FDR)
			250	250			
Barrier function	*CLDN15*	1[b]	2.30[a]	2.39[a]	10.535	0.001	0.003
	MUC2	1[b]	2.83[a]	2.23[a]	11.597	< 0.001	0.002
	OCLN	1[a]	0.75[b]	0.72[b]	5.604	0.012	0.030
	TFF3	1[b]	4.74[a]	4.34[a]	30.806	< 0.001	< 0.001
	ZO1	1[a]	0.72[b]	0.63[c]	33.595	< 0.001	< 0.001
Immune response	*CCL20*	1[a]	0.31[b]	0.56[ab]	12.355	0.041	0.071
	CXCL2	1[a]	0.53[b]	0.54[b]	8.514	0.025	0.050
	HSPA4	1[a]	0.87[ab]	0.73[b]	28.923	0.012	0.030
	IFN-γ	1[a]	0.42[b]	0.54[b]	11.792	0.030	0.057
	IFNGR1	1[a]	0.87[ab]	0.80[b]	3.801	0.044	0.074
	IL-6	1[a]	0.55[b]	0.33[b]	6.152	0.001	0.003
	IL-8	1[a]	0.48[b]	0.45[b]	4.530	0.022	0.047
	TGF-β1	1[a]	0.83[b]	0.79[b]	10.668	0.017	0.040
	TLR2	1[b]	1.93[a]	1.65[a]	3.722	0.018	0.040
Nutrient transport	*SLC16A1/MCT1*	1[b]	1.23[a]	0.90[b]	4.412	0.002	0.006
	SLC30A1/ZnT1	1[a]	0.54[b]	0.51[b]	5.546	< 0.001	< 0.001
	SLC39A4/ZIP4	1[b]	1.43[a]	1.84[a]	4.262	0.001	0.005
	SLC5A1/SGLT1	1[b]	2.11[a]	2.79[a]	3.625	< 0.001	0.001
Enzyme/Hormone	*ANPEP*	1[a]	0.39[b]	0.34[b]	4.626	< 0.001	0.002
	CCK	1[a]	0.69[b]	0.64[b]	10.469	0.002	0.006
	DAO1	1[b]	3.60[a]	3.64[a]	4.953	< 0.001	0.000
	HNMT	1[a]	0.74[b]	0.70[b]	4.925	< 0.001	0.002
	IGF1R	1[b]	1.41[a]	1.44[a]	8.580	0.040	0.071
	PYY	1[b]	1.31[ab]	1.84[a]	23.337	0.023	0.048
	SI	1[a]	0.56[b]	0.78[ab]	9.335	0.008	0.024
	SOD2	1[b]	1.93[a]	1.50[a]	14.961	0.001	0.003

[1]Data are means of 8 observations per treatment. Gene abundance values are presented as ratios of cycle relative threshold value for each gene normalized to that of the reference sample.

[2]*CLDN15*: claudin 15; *MUC2*: mucin 2; *OCLN*: occludin; *TFF3*: trefoil factor 3; *ZO1*: zonula occludens 1; *CCL20*: chemokine (C-C motif) ligand 20; *CXCL2*: chemokine (C-X-C motif) ligand 2; *HSPA4*: heat shock protein 70; *INF-γ*: interferon gamma; *IFNGR1*: interferon gamma receptor 1; *IL-6*: interleukin 6; *IL-8*: interleukin 8; *TGF-β1*: transforming growth factor beta 1; *TLR2*: toll-like receptor 2; *SLC16A1/MCT1*: monocarboxylate transporter 1; *SLC30A1/ZnT1*: solute carrier family 30 (zinc transporter) member 1; *SLC39A4/ZIP4*: solute carrier family 39 (zinc transporter) member 4; *SLC5A1/SGLT1*: solute carrier family 5 (sodium/glucose cotransporter) member 1; *ANPEP*: aminopeptidase-N; *CCK*: cholecystokinin; *DAO1*: diamine oxidase; *HNMT*: histamine N-methyltransferase; *IGF1R*: insulin-like growth factor 1 receptor; *PYY*: peptide tyrosine tyrosine; *SI*: sucrase-isomaltase; *SOD2*: superoxide dismutase

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

[a-b] Means with different subscripts within a row differ ($P < 0.05$)

3.4. Microbiota 16S rRNA gene analysis

For an assessment of the effects of Cu supplementation on large intestinal microbiota, the 16S rRNA of colon microbiota was determined. Alpha (Shannon, Simpson, and Inverse Simpson index) and beta diversity metrics were used to estimate diversity among microbial communities. There were no differences in alpha diversity indices ($P > 0.1$, data not shown), and beta diversity among experimental treatments was not different either ($P_{ENVFIT} = 0.4$, data not shown).

In the colon microbiota, 17 operational taxonomic units representing the phyla Actinobacteria, Bacteroidetes, Chlamydiae, Cyanobacteria, Deferribacteres, Epsilonbacteraeota, Euryarchaeota, Fibrobacteres, Firmicutes, Kiritimatiellaeota, Lentisphaerae, Patescibacteria, Planctomycetes, Proteobacteria, Spirochaetes, Synergistetes, and Tenericutes were identified (Figure 12. a). Firmicutes and Bacteroidetes were present at a mean relative abundance of ≥30% (average 52% and 30% respectively), followed by Spirochaetes (average 3.40%) and Tenericutes (average 3%). The majority of Firmicutes phylum corresponded to *Ruminococcaceae* (average 26.8%), *Erysipelotrichaceae* (average 8.2%), *Lachnospiraceae* (average 6.2%), *Acidaminococcaceae* (average 3.8%), *Veillonellaceae* (average 3.6%), and *Christensenellaceae* (0.8-1.4%). The majority of Bacteroidetes phylum corresponded to *Prevotellaceae* (average 16.8%), *Rikenellaceae* (5.7-8.1%), *Muribaculaceae* (average 5.2%), and most Spirochaetes belonged to the *Spirochaetaceae* family (1.5-5.5%), as illustrated in Figure 12. b.

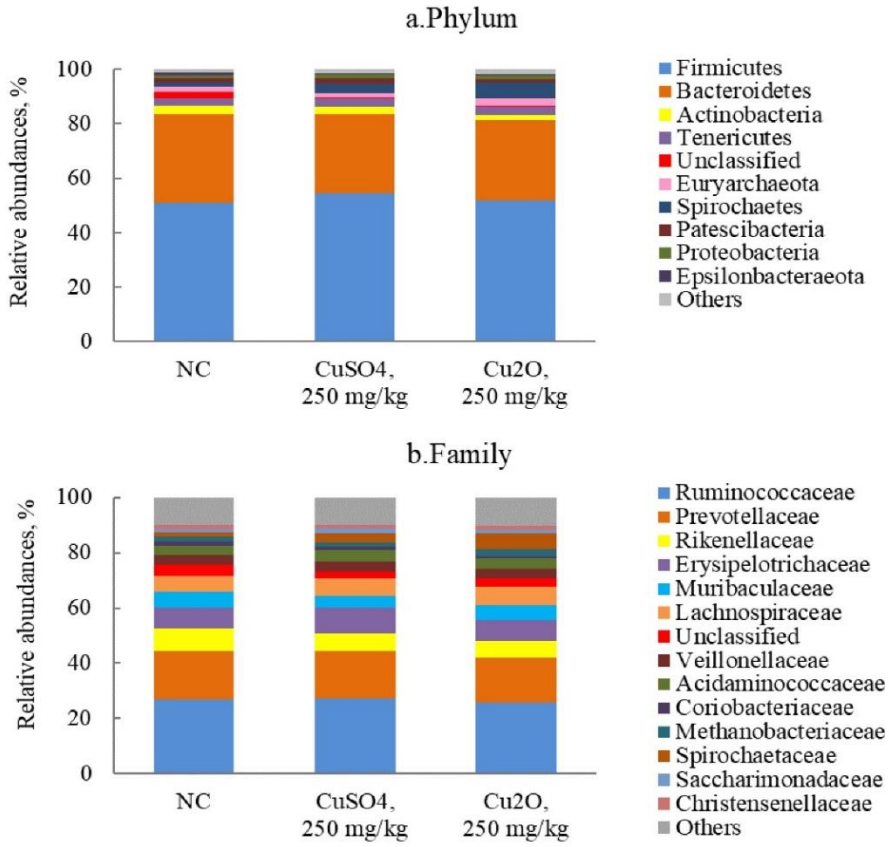

Figure 12. Relative abundance (%) of phyla (a), and families (b) present in the colon microbiota of pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O. The rest of the taxonomic groups are pooled together (those representing less than a mean of 1% of phyla and families).

The following families were different (Figure 13) among dietary treatments: *Rikenellaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Spirochaetaceae*, *Christensenellaceae*, and *Peptostreptococcaceae*. Some significances at the genus level i.e. *Holdemanella*, *Campylobacter*, and *Streptococcus* have been also observed.

Pigs fed 250 mg Cu/kg from $CuSO_4$ had a greater ($P < 0.05$) relative abundance of *Erysipelotrichaceae* (0.28 fold increase), *Lachnospiraceae* (0.26 fold increase), *Spirochaetaceae* (1.1 fold increase), and *Peptostreptococcaceae* (1.31 fold increase) families, and *Holdemanella* (0.34 fold increase) genera compared with pigs fed a diet not supplemented with Cu. Pigs fed 250 mg Cu/kg from $CuSO_4$ had lower ($P < 0.05$) levels of

Rikenellaceae (0.3 fold decrease), *Christensenellaceae* (0.79 fold decrease) families, and *Campylobacter* (0.82 fold decrease), and *Streptococcus* (1.57 fold decrease) genera than the NC.

Supplementing 250 mg Cu/kg from Cu_2O to the diet increased ($P < 0.05$) relative abundance of *Lachnospiraceae* (0.31 fold increase), *Spirochaetaceae* (1.90 fold increase), and *Peptostreptococcaceae* (0.97 fold increase) families compared with pigs fed the non-supplemented diet. In contrast, Cu_2O supplementation decreased ($P < 0.05$) the relative abundance of the *Rikenellaceae* family (0.51 fold decrease), and genera like *Holdemanella* (0.9 fold decrease), *Campylobacter* (1.28 fold decrease), and *Streptococcus* (0.58 fold decrease) compared with pigs fed the NC diet.

Pigs fed the diet with 250 mg Cu/kg from $CuSO_4$, tended to have increased abundance of *Rikenellaceae* (0.22 fold increase, $P = 0.088$), and *Erysipelotrichaceae* (0.22 fold increase, $P = 0.068$) families, and increased ($P < 0.05$) the abundance of *Holdemanella* (1.24 fold increase) genera, compared with pigs fed 250 mg Cu/kg from Cu_2O.

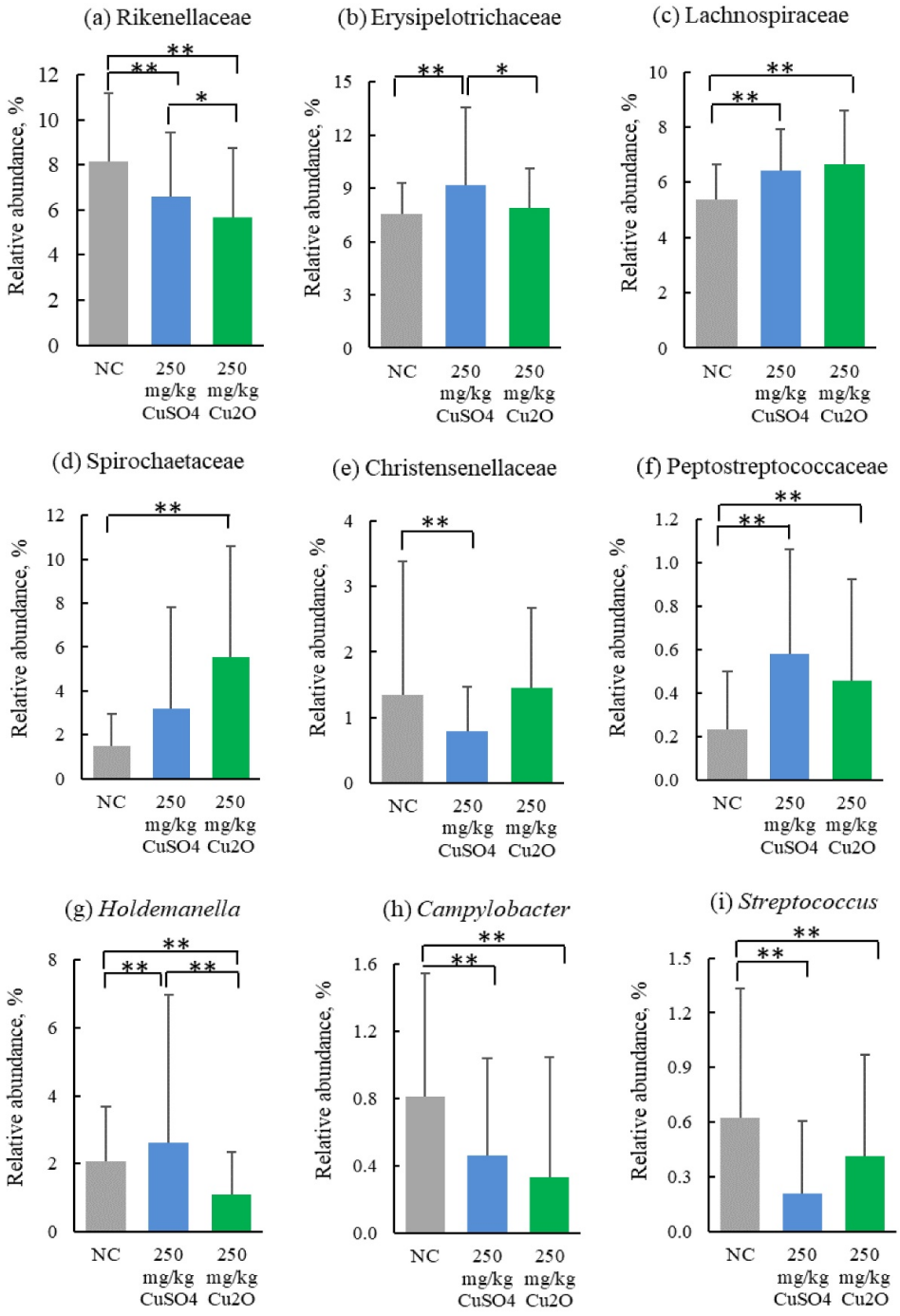

Figure 13. Colonic bacterial families and genera whose abundance significantly differs between pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O. Data are means of 8 observations per treatment. Error bars show the standard deviation. ANOVA significance, *indicates $P < 0.1$, and ** $P < 0.05$.

4. Discussion

This work was part of a larger study that has been previously published (Blavi et al., 2021) and is recommended as complementary information. The previous study reported increased BW by supplementation of diets with 250 mg/kg of Cu, specifically Cu_2O, starting from the end of phase one. At the same time, pigs fed 250 mg Cu/kg from Cu_2O had lower Cu concentration in the liver and spleen compared with those fed 250 mg Cu/kg from $CuSO_4$. Therefore, we decided to take advantage of the previously collected samples at the end of phase one to elucidate the differences between the Cu sources and the NC.

4.1. Effects of copper on the jejunum gene abundance

The integrated intestinal barrier is critical for epithelial cell function as well as for preventing harmful microorganisms from passing through the mucosa (Hu et al., 2012). *CLDN15* is a tight junction barrier protein (Tamura et al., 2008), which is important for small intestinal sugar absorption due to its cation-selective channel property (Tamura et al., 2011). *MUC2* is the key component of the mucus gel layer in the gut mucosa, which is secreted by goblet cells (Li, 2011) and protects against luminal viral infection as an essential structural component of the intestinal mucin layer (Deplancke and Gaskins, 2001). *TFF3* is important for repairing the intestinal mucosa (Liu et al., 2021). Therefore, the observation that therapeutic doses of Cu increased the abundance of *CLDN15*, *MUC2*, and *TFF3* may indicate that dietary Cu may help improve intestinal barrier function. Upregulation of *TFF3* and *CLDN15* coincides with better growth performance in weanling pigs (González-Solé et al., 2020).

One of the roles of Cu is aiding the improvement of animals' innate and acquired immunological functions (Prohaska and Failla, 1993). The immune system is triggered when it is exposed to pathogenic or nonpathogenic antigens, resulting in the release of cytokines such as TNF-α, IL-1, and IL-6 (Al-Sadi et al., 2009). The reduced abundance of the immune response proteins *CXCL2*, *IFN-γ*, *IL-6*, *IL-8*, and *TGF-β1* that was observed in pigs fed diets containing therapeutic levels of Cu ($CuSO_4$ or Cu_2O) also indicates an improved immune response. *CXCL2* is a chemoattractant for neutrophils (Kielian et al., 2001) and is important for the inflammatory response and growth regulation (Kim et al., 2010). Pro-inflammatory cytokines including *IFN-γ*, *IL-6*, and *IL-8*, enhance intestinal epithelial permeability and provoke a pathologic opening of the intestinal tight junction barrier (Al-Sadi et al., 2009). The reduced mucosal *IFN-γ*, *IL-6*, and *IL-8* in the presence of high levels of Cu indicate that therapeutic levels of Cu can minimize intestinal inflammation thus minimizing tissue damage and reducing gut permeability. These results agree with Song et al. (2013) who also showed reduced mucosal *IL-6* and *TNF-α* and improved mucosal barrier integrity of weanling pigs fed a diet with increased Cu. The reduced concentration of the anti-inflammatory cytokine *TGF-β1*, which protects against intestinal inflammation (Howe et al., 2005), with Cu supplementation may be a result of a low concentration of pathogens and low intestinal inflammation in the pigs used in this experiment.

The fact that supplementation of diets with 250 mg/kg of Cu upregulated the abundance of *SLC5A1/SGLT1* may indicate that pigs fed the diets containing therapeutic levels of Cu may have had improved absorption of glucose. *SLC5A1/SGLT1* is the main intestinal glucose transporter (Röder et al., 2014) that transports glucose into the enterocytes from the lumen.

The downregulation of *CCK* and upregulation of *PYY* were observed for pigs fed diets with elevated concentrations of Cu. *CCK* is a brain-gut peptide (Strader and Woods, 2005) that inhibit feed intake and satiety (Moran and Kinzig, 2004) so a reduction of *CCK* may have the opposite effect. The hormone *PYY* is primarily produced and released by endocrine L-cells in the distal region of the gastrointestinal tract in response to feed intake (Karhunen et al., 2008). Because *PYY* mediates ileal and colonic brakes, which slow down gastric emptying and stimulate digestive processes to promote nutrition absorption (Pironi et al., 1993) while reducing appetite (Sleeth et al., 2010). Although we only analyzed the gene expression at the end of phase one, long-term regulation of these hormones may be one of the reasons behind the increased feed intake in pigs fed the diet containing Cu_2O throughout the experiment (Blavi et al., 2021).

Insulin-like growth factor 1 (*IGF1*) has many growth-promoting and metabolic activities (Froesch, 1985), and is an essential regulator of intestinal cell growth and differentiation (Jones and Clemmons, 1995). Increased abundance of *IGF1* and *IGF1R* in piglet small intestine enhances growth performance (Li et al., 2006). The upregulation of *IGF1R* that was observed in pigs fed diets with added Cu, therefore, might have been associated with the increased growth performance of these pigs. The downregulation of digestive enzyme SI, which was caused by $CuSO_4$, but not by Cu_2O, may have restricted the absorption of glucose and free AA. Feeding high levels of Cu may result in an increased abundance of antioxidant-related mRNA *SOD2* (Huo et al., 2021; Li et al., 2021), and a reduced SOD level is likely to result in an increased ROS level. Therefore, the upregulation of *SOD2* that was observed in pigs fed the diets with 250 mg/kg of Cu may be considered an anti-inflammatory response by Cu.

Overall, the intestinal gene abundance data from the experiment indicate that the improved growth performance by pigs fed diets with therapeutic levels of Cu (Blavi et al., 2021), maybe a result of improvement in intestinal epithelial barrier function, modulation of immunological and inflammatory responses, and increased feed intake and nutrient absorption.

4.2. Effects of copper on the colon microbiota profile

Therapeutic levels of Cu alter the intestinal microbiota in poultry (Pang et al., 2009; Forouzandeh et al., 2021) and swine (Wang et al., 2012; Villagómez-Estrada et al., 2020). Copper supplementation may act by reducing the total pathogenic organism in the gut (Xia et al., 2004) and reducing susceptibility to disease due to its antimicrobial effect. In fact, one of the possible mechanisms by which Cu may promote growth in animals is restricting the growth of microbes in the intestinal tract (Espinosa et al., 2019) and increasing nutrient absorption (Villagómez-Estrada et al., 2020).

Members of the *Lachnospiraceae* family produce butyric acid via fermentation of polysaccharides (Quan et al., 2018), and its abundance is positively correlated with energy metabolism (Y. Zhang et al., 2019) and improved feed efficiency in pigs (Yang et al., 2017; Quan et al., 2018) and poultry (Stanley et al., 2016). The role of the *Spirochaetaceae* family is not completely clear, but some of its members have greater relative abundance in low residual feed intake (RFI) pigs compared with high RFI (McCormack et al., 2017). The *Peptostreptococcaceae* family is present in a higher proportion in the gut microbiota of healthy animals than of animals experiencing dysbiosis of the intestinal microbiota (Fan et al., 2017). Therefore, the fact that at the family level, the relative abundance of *Lachnospiraceae*, and *Peptostreptococcaceae*

increased with high levels of $CuSO_4$ or Cu_2O, and the relative abundance of *Spirochaetaceae* increased with high levels of Cu_2O, may indicate that changes in the microbiota contributed to a greater growth performance of pigs fed the Cu supplemented diets. However, the implication of the reduced concentration of the family *Christensenellaceae* in feces from pigs fed diets with high concentrations of Cu is less certain because the relationship between the abundance of *Christensenellaceae* and gut health remains unclear.

An increase in the concentration of favorable families in the gut may result in a reduction of potentially pathogenic organisms. The reduced abundance of *Rikenellaceae* and *Campylobacter* and *Streptococcus*, therefore, may result in reduced intestinal disease as has been demonstrated in mice (He et al., 2019).

Overall, the increase in beneficial families and the reduction of pathogens in the gastrointestinal tract of pigs fed diets with elevated concentrations of Cu possibly improved intestinal nutrient absorption and contributed to the increased growth performance observed in the experiment (Blavi et al., 2021). The reduced concentration of pathogens also supports the lower abundance of immune response genes that were observed in pigs fed the diets with therapeutic doses of Cu. This observation agrees with similar modulations of the microbiota in pigs fed a diet containing 160 mg/kg of Cu from $CuSO_4$ or Cu hydroxychloride, which resulted in improved growth performance (Villagómez-Estrada et al., 2020). The observation that Cu_2O seemed to be more efficient than $CuSO_4$ in terms of reducing the growth of *Rikenellaceae* family and *Holdemanella* genus may indicate that Cu_2O promotes intestinal health to a greater degree than $CuSO_4$. Similar observations were reported from a study with broiler chickens (Forouzandeh et al., 2021).

4.3. Effects of copper on oxidation and inflammation

Although Cu plays a pivotal role for some key enzymes, the addition of therapeutic levels of Cu (150–250 mg Cu/kg) results in oxidation and catalyzing of hydroxyl radical forms (Gaetke and Chow, 2003). One of the metabolic products of lipid peroxides is MDA, which is generated by oxygen-free radicals in tissues (Zhan et al., 2006). A high concentration of MDA is an indicator of oxidation and has been identified in the liver (Pu et al., 2016) and duodenal mucosa (Huang et al., 2015) of pigs fed diets with high Cu concentrations. The observation that liver MDA in pigs fed the diet with 250 mg Cu/kg from $CuSO_4$ was approximately 12 % greater than in the liver of pigs fed NC or the diet with Cu_2O supplementation to some extent agrees with the tendency in reduction of the concentration of the antioxidant enzyme GSH-Px. These results are consistent with the fact that there is greater prooxidant activity in the liver of broiler chickens and pigs fed a diet containing $CuSO_4$ compared with those fed a diet containing tribasic Cu chloride (Miles et al., 1998; Luo et al., 2005; Fry et al., 2012). The chemical characteristics of Cu could be one of the reasons behind this difference. Unlike $CuSO_4$ which is highly water-soluble (Pang and Applegate, 2006), CuO_2 is not soluble in water (Baker, 1999). Since only soluble compounds can be absorbed in the small intestine (Wapnir, 1998) Cu solubility plays an important role in its absorption. Therefore, the high solubility of $CuSO_4$ may explain the greater accumulation of Cu in the liver of pigs fed $CuSO_4$ compared with pigs fed CuO_2 or NC (Blavi et al., 2021) which may eventually result in the greater oxidative effect of $CuSO_4$.

The observation that 250 mg Cu/kg from $CuSO_4$ tended to increase serum IL-1β and increased TNF-α may be another consequence of the greater accumulation of Cu in pigs tissues (Blavi et al., 2021). Oxidative effects of excess Cu can induce inflammation (Song

et al., 2013). Pro-inflammatory cytokines including IL-6, TNF-α, and INF-α disrupt the intestinal barrier, allowing luminal antigens to penetrate deeper into the tissue (Capaldo and Nusrat, 2009). During hepatic toxic injury, pro-inflammatory cytokines such as TNF-α and IL-1β are released into the bloodstream from the liver (Lacour et al., 2005). As a consequence, cytokine signals produced in response to tissue damage may be used as biomarkers for cellular responses to hepatotoxicity and inflammation. Indeed, elevated serum levels of TNF-α and IL-1β have been reported in patients with liver injuries (Lacour et al., 2005; Mannaa and Abdel-Wahhab, 2016). Moreover, serum concentration of pro-inflammatory cytokine TNF-α, which is one of the primary defenders against copper poisoning, increased with the addition of 240 mg Cu/kg in rats diet (Zhang et al., 2017). The elevated levels of IL-1β and TNF-α with therapeutic doses of Cu from $CuSO_4$ in this experiment may indicate a general increase in inflammation. However, more research is needed to determine whether elevated levels of cytokines are directly linked with high Cu concentration and whether their alteration eventually leads to long-term weight gain.

5. Conclusion

In conclusion, supplementation of diets for growing pigs with Cu at therapeutic levels (250 mg Cu/kg) improved the intestinal barrier function and modulated the intestinal immune responses by regulating several inflammatory cytokines. Dietary Cu increased the concentrations of microbial populations that are favorable to growth, immunity, and gut health. However, $CuSO_4$, but not Cu_2O, increased liver oxidation and biomarkers for inflammation in serum.

MANUSCRIPT III

Limited impacts of high doses of dietary copper on the gut bacterial metal resistome explain negligible co-selection of antibiotic resistance

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(Under Second Revision)

SUMMARY

High dietary intake of Cu has previously been linked to the selection of Cu resistance and co-selection of antibiotic resistance in specific gut bacteria. Based on a novel HT-qPCR metal resistance gene chip as combined with 16S rRNA gene amplicon sequencing and phenotypic resistance typing of *Escherichia coli* isolates, we here report the impacts of two contrasting Cu-based feed additives on the swine gut bacterial metal resistome and community assembly. DNA was extracted from fecal samples (*n* = 80) collected at day 26 and 116 of the experiment from 200 pigs allotted to five dietary treatments: negative control (NC) diet with 20 μg $CuSO_4$ g^{-1} and four diets added 125 or 250 μg $CuSO_4$ g^{-1} feed or 125 or 250 μg Cu_2O g^{-1} feed to the NC diet. Dietary Cu supplementation reduced the relative abundance of *Lactobacillus*, but it had negligible impacts on bacterial community composition relative to the gut microbiome maturation effect (time). The relative importance of different bacterial community assembly processes was not markedly affected by the dietary Cu treatments, and differences in swine gut metal resistome composition could be explained primarily by differences in bacterial community composition rather than by dietary Cu treatments. High dietary Cu intake (250 μg Cu g^{-1}) selected for phenotypic Cu resistance in *E. coli* isolates, but surprisingly it did not result in increased prevalence of the Cu resistance genes targeted by the HT-qPCR chip. In conclusion, the lacking impacts of dietary Cu on the gut bacterial metal resistome explain results from a previous study showing that even high therapeutic doses of dietary Cu did not cause co-selection of antibiotic resistance genes and mobile genetic elements known to harbor these genes.

1. Background

Antibiotic growth promoters have been widely used in swine production for many years (Dibner and Richards, 2005), but this agricultural practice has been banned, or at least tightly regulated, in many countries during recent years due to risks for the development and transfer of antibiotic resistance between animals and humans (You and Silbergeld, 2014; Zhao et al., 2021). Consequently, there is a need for alternative growth-promoting compounds. Metals such as zinc (Zn) and copper (Cu) have received considerable attention as alternative growth promoters in pigs due to their antimicrobial activities (Højberg et al., 2005). Zn is primarily supplemented for weaners resulting in short-term supplementarion in high doses, whereas Cu is widely used in fattener diets resulting in long-term Cu supplementation.

The physiological Cu requirement for swine is 5 to 6 µg Cu g^{-1} diet (NRC, 2012), and the maximum dosage authorized in the EU is 25 µg Cu g^{-1}, with an exception of 150 µg Cu g^{-1} in case of suckling and weaning pigs up to 4 weeks after weaning (European Commission, 2018). However, in many non-EU countries, the commercial practice generally uses therapeutic doses of Cu (150 to 250 µg Cu g^{-1}) in swine diets as a growth promoter. Numerous studies have confirmed the growth-promoting effect of Cu in swine production (Espinosa et al., 2017; Espinosa and Stein, 2021; Pérez et al., 2011). The underlying animal growth-promoting mechanisms of dietary Cu are not yet fully understood, but several modes of action have been proposed (Espinosa and Stein, 2021). Cu speciation influences the biological activity of Cu with Cu^{+} being more toxic than Cu^{2+} (Rensing et al., 2018; Popov et al., 2020). Although Cu speciation will be affected during animal gut passage, recent animal feeding trials have demonstrated that monovalent copper oxide (Cu_2O) showed superior effects on animal performance and

gut microbiome composition as compared to copper (II) sulfate ($CuSO_4$) (Blavi et al., 2021; Forouzandeh et al., 2021; Hamdi et al., 2018).

Animal gut bacteria can easily develop resistance to Cu and other metallic growth promoters (e.g. zinc) via adaptive mutations or acquisition of metal resistance genes (MRGs) by horizontal gene transfer (Aarestrup and Hasman, 2004; Hasman et al., 2005, 2006). Metal resistance genes are commonly located on plasmids or other mobile genetic elements (MGEs) and the use of elevated dietary Cu diets in pigs can thus be associated with a risk for co-selection (co-resistance) of antibiotic resistance genes (ARGs) (Hasman et al., 2005; Yazdankhah et al., 2014). Co-selection of MRGs and ARGs may also happen by cross-resistance or co-regulation (Baker-Austin et al., 2006). We recently reported the first controlled study investigating the impact of two contrasting Cu-based feed additives (growth promoters) on the overall swine gut microbiome and antibiotic resistome and found no evidence for the co-selection of ARGs (Brinck et al., 2023). However, we did not infer if the high-Cu diets (250 μg Cu g^{-1} feed) were indeed high enough to directly select for Cu resistance in the gut bacteria or whether co-selection of Cu and antibiotic resistance could still have happened in specific culturable bacterial species within the microbiome. Understanding these issues is important for a more complete characterization of the risks for Cu-induced co-selection of ARGs in pigs.

Very recently, a novel HT-qPCR metal resistance gene chip (MRG chip) has been made available to the scientific community (Zhu et al., 2022). The MRG chip allows for comprehensive quantification of known prokaryotic Cu resistance genes (CRGs) and other MRGs, and we, therefore, decided to make a follow-up study using frozen fecal matter samples from our previous studies (Blavi et al., 2021; Brinck et al., 2023). Compared to our previous antibiotic resistome study targeting ARGs (Brinck et al., 2023),

we this time used the MRG chip to study the ability of dietary Cu to select for CRGs and other MRGs thereby shaping the gut bacterial metal resistome. In addition, we looked into the impacts of dietary Cu on the bacterial community assembly processes shaping the pig gut microbiome using iCAMP (Ning et al., 2020) and we used a cultivation-dependent approach targeting *Escherichia coli* to complement the HT-qPCR ARG data from our first study. Specifically, we generated the following hypotheses for our study: high-Cu diets (125 or 250 µg Cu g^{-1} feed) will affect bacterial community composition, diversity and underlying community assembly processes as revealed by 16S rRNA gene amplicon sequencing; high-Cu diets will increase the abundance of known genes that have been associated with Cu resistance in gut bacteria; high-Cu diets will co-select for genes conferring resistance to other metals; high-Cu diets will select for phenotypic Cu resistance in *E. coli* isolates; Cu resistant *E. coli* isolates are more resistant to contrasting classes of antibiotics than corresponding Cu susceptible isolates are (i.e. co-selection of Cu and antibiotic resistance).

2. Materials and methods

2.1. Animal management and samples collection

Comprehensive information about the pig feeding trial with 200 growing pigs has been described by Blavi et al. (2021). In brief, the study involved 6-weeks old growing pigs with an average initial body weight of 11.5 ± 0.98 kg that were assigned to a randomized complete block design. Pig fecal matter was obtained on day 26 and 116 of the experiment (direct sampling from the rectum; 8 fecal samples per treatment group) as described previously (Brinck et al., 2023). Sampling time points were selected to get

data on the effects of dietary Cu both during the initial animal growth stage (day 26) and shortly before animals would normally be sent for slaughter (day 116), when microbiomes were suspected to be most selected by the dietary Cu treatments. Fecal samples were immediately placed into liquid nitrogen and stored at -80^{o} C. The dietary treatments included a negative control (NC) diet with 20 µg Cu g^{-1} ($CuSO_4$), as well as four diets added 125 µg Cu g^{-1} feed ($CuSO_4$ or Cu_2O) or 250 µg Cu g^{-1} feed ($CuSO_4$ or Cu_2O) on top of the NC diet. For short, the dietary Cu treatments were named 125 $CuSO_4$, 250 $CuSO_4$, 125 Cu_2O, and 250 Cu_2O, respectively.

2.2. Fecal DNA extraction

DNA was extracted from 250 mg thawed fecal samples (*n* = 80) using the DNeasy PowerSoil Pro DNA Isolation Kit (Qiagen, Germany) following the manufacturer's instructions. Nanodrop-1000 spectrophotometer (ThermoFisher Scientific, USA) and Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) were used to assess the quality and quantity of the isolated DNA. DNA concentrations varied between 50-280 ng $µl^{-1}$ for the fecal samples. All DNA extracts had A260/A280 ratios greater than 1.8. Extracted DNA was stored at -80°C until used for 16S rRNA gene amplicon sequencing and HT-qPCR array.

2.3. 16S rRNA gene amplicon sequencing and data processing

The primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the V4 region of the bacterial 16S rRNA gene by PCR (Walters et al., 2016). At Novogene Bioinformatics Technology Co. Ltd. (UK), a small-fragment library was created, followed by paired-end sequencing with

the Novaseq PE250. The DADA2 pipeline (Callahan et al., 2016) in R version 4.0.5 was used to process the 16S rRNA gene amplicon sequences. Filtering and trimming of the reads were done using the default settings of DADA2. The paired-end reads were subsequently combined, and an amplicon sequence variant (ASV) database was built using the core sample inference algorithm and trained error models of DADA2 followed by the removal of chimeras. The taxonomy of the ASVs was assigned using the Silva 138.1 prokaryotic SSU taxonomic training data (Quast et al., 2013) formatted for DADA2. A phylogenetic tree was constructed based on the ASVs by using MAFFT (Katoh et al., 2002) and FastTree (Price et al., 2010). For downstream analysis, ASVs identified as mitochondrial DNA or chloroplast DNA were removed, and all samples were rarefied to an even sampling depth of 62,000 which was the minimum number of reads in all samples resulting in a total of 4,164 ASVs.

2.4. High-throughput qPCR and data processing

High-throughput qPCR reactions were performed by the Wafergen SmartChip Real-time PCR system (ThermoFisher, USA), using a total of 86 validated primer sets targeting MRGs associated with Ag, Cu, Hg, Ni, Zn, Zn/Cd/Co/Pb, and MDR as well as MGEs (Table S1) according to Zhu et al. (2022). Out of the 86 gene targets assayed, 84 were detected targeting 45 MRGs and 8 MGEs. The detected MRGs were mostly associated with Zn/Cd/Co/Pb (17 primers targeting 9 genes), Cu (14 primers targeting 11 genes), and Zn (14 primers targeting 8 genes; Fig. S1A). Efflux pump, gene regulation, and uptake prevention were the most prevalent resistance mechanisms among all MRGs (91%; Fig. S1B) and the detected MGEs only encoded integrases (4 primers) and transposases (9 primers; Fig. S1C).

All qPCR reactions were carried out in technical triplicates, with a non-template control included in each run. A threshold cycle (C_T) of 31 was used as the detection limit for the individual PCR reactions. C_T values higher than 31 were set to 0 and only genes detected in all triplicates were regarded as positive. Relative gene copy numbers were calculated with the formula: relative gene copy numbers = $10^{(31-Ct)/(10/3)}$ as described previously (Looft et al., 2012), where Ct refers to HT-qPCR results. HT-qPCR data were normalized among samples by dividing the relative gene copy numbers by the corresponding 16S rRNA gene copy numbers.

2.5. Isolation and characterization of bacteria

In order to compare our data to our previous HT-qPCR ARG study (Brinck et al., 2023), bacterial isolates were exclusively obtained from fecal samples (*n* = 24) collected from the NC, 250 $CuSO_4$, and 250 Cu_2O treatments at the end of the experiment (d 116). Fecal samples were extracted by shaking 1 g of feces with 10 ml of 0.9% sterile NaCl for 1 h on a rotary shaker (150 rev per min, 25°C). Relevant dilutions of the liquid phase were prepared and spread in triplicates on Reasoner's 2A (R2A) agar plates (Reasoner and Geldreich, 1985). R2A medium was used, as the isolation campaign was part of a larger study also involving cultivation of bacteria from different aquatic and terrestrial environments. R2A plates were incubated for 3 d at 25°C. After incubation, isolates (*n* = 462) were randomly picked from the plates and purified twice by re-streaking to obtain pure colonies. The isolates were stored at -80°C in R2A containing 30% (v/v) glycerol.

DNA was first extracted from 95 random isolates (treatments: NC, 250 $CuSO_4$, and 250 Cu_2O) grown on R2A agar plates using the Q-Extract DNA Extraction Solution (AMPLIQON, Odense, Denmark) according to the instruction manual. Isolates were

subsequently identified by Sanger sequencing, which was conducted at Eurofins Genomics (Germany) using the 27F 16S rRNA gene primer. Most isolates were identified as *E. coli* (Table S2), and this species was therefore chosen as an indicator organism for our study. To identify *E. coli* isolates among the remaining isolates (*n* = 462), all were cultivated on *E. coli*-specific m-TEC ChromoSelect agar plates (Sigma-Aldrich, Germany) and incubated at 37°C for 22–24 h. To verify the specificity of the m-TEC ChromoSelect agar, ten of the isolates capable of growing on the medium were randomly selected for Sanger sequencing and they were all identified as *E. coli*. Sixty-one percent of the 462 isolates were in this way identified as *E. coli* (*n* = 284; Fig. S2A). Out of the identified *E. coli* isolates, 17.6% were from the NC group, whereas 36.3% and 46.1% belonged to the 250 $CuSO_4$ and 250 Cu_2O treatments, respectively (Fig. S2B).

2.6. Phenotypic determination of Cu and antibiotic resistance

All *E. coli* isolates (*n* = 284) were typed for Cu and antibiotic resistance as described previously (Berg et al., 2010). In brief, all isolates were cultivated on R2A agar plates containing either $CuSO_4$ (1 mM), ampicillin (32 mg/L), chloramphenicol (32 mg/L), colistin (64 mg/L), nalidixic acid (32 mg/L), streptomycin (32 mg/L), and tetracycline (16 mg/L). Resistance to the compounds was scored by visual growth inspection. The antibiotics were selected to represent contrasting classes of antibiotics with different modes of action. Antibiotics were obtained from VWR chemicals (Pennsylvania, USA). Isolates were defined as multidrug-resistant (MDR) when they were resistant to at least two of the screened antibiotics.

2.7. Statistical analyses

R version 4.0.5 (2021-03-31) was used for data exploration, graphics, and all statistical analyses. Bacterial community composition was examined with "microeco" version 0.10.1 and "ggplot2" version 3.3.3 R packages (Liu et al., 2021; Wickham, 2016). Alpha diversity of samples was displayed with Chao1 and Shannon measures, and statistical differences between treatment groups and time points were assessed by the Kruskal-Wallis rank sum test (Kruskal and Wallis, 1952) combined with Dunn's test for Multiple comparisons. Beta diversity of samples was displayed using Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity metrics. Differences in bacterial community composition among treatment groups and time points were investigated by perMANOVA. Differential abundance of ASVs was assessed among the different treatment groups applying analysis of compositions of microbiomes with bias correction with default settings (ANCOM-BC; Lin and Peddada, 2020).

The relative importance of different bacterial community assembly processes was investigated by iCAMP (Infer Community Assembly Mechanisms by Phylogenetic-bin-based null model analysis) as described previously (Ning et al., 2020). ASVs were initially divided based on their relative abundance. ASVs constituting < 0.1% in all samples were considered "rare", whereas ASVs with a relative abundance > 1% in one or more samples were considered "abundant"; remaining ASVs were classified as "intermediate" (Zhang et al., 2020). Dispersal limitation, homogenizing dispersal, and drift fractions are primarily regarded as the stochastic process according to iCAMP null model theory and Quantifying assembly Processes based on Entire-community Null model analysis (Zhou and Ning, 2017).

To examine the potential effects of Cu on the bacterial community structure and

MRG/MGE composition, data from pigs with the same treatment and sampling time point were aggregated. MRG/MGE profiles were analyzed using the “vegan” R package version 2.6-2 (Oksanen et al., 2022). The function *adonis* was used to analyze variations in MRG/MGE compositions across treatment groups and time points using permutation multivariate analysis of variance (perMANOVA; Anderson, 2001). NMDS ordination using Bray-Curtis dissimilarity index was used to display dissimilarities of MRG/MGE compositions across treatments and time points. The "pheatmap" R package version 1.0.12 was used to create a heatmap of relative gene abundances. Significant differences in the relative abundance of MRG or MGE classes and also in individual genes were assessed by a one-way Analysis of Variance (ANOVA) test using the “dplyr” R package version 1.0.8. The *protest* function was used to complete the Procrustes test for correlation analysis between bacterial communities and MRG/MGE composition based on NMDS ordinations.

Logistic regression with binomial distribution using the generalized linear model (GLM) function *glm* was used to do an ANOVA-type analysis of Cu and antibiotics resistance in isolates using treatment as a distinguishing factor. The model was subsequently compared against a model not distinguished by treatment and the significance of the models was tested using the Chi-square test.

3. Results

3.1. Impacts of high Cu diets on gut bacterial community composition and assembly

After quality filtering, the 80 fecal samples yielded a total of 6,166,891 paired-end 250-bp sequences, ranging between 62,280 and 96,631 sequences per sample. After

rarefication, the 4,164 ASVs that were formed from the sequences were allocated to 25 distinct phyla and 463 genera. *Firmicutes* comprised the dominant phylum among all treatments and time points presented at a mean relative abundance of 91.5 % followed by *Bacteroidota,* and *Euryarchaeota* both with a mean relative abundance of 2.5 % (Fig. S3). *Clostridium sensu stricto 1* was the most abundant genus representing up to 21.5% of all ASVs at the first time point and 47.10% at the second time point (Fig. S4).

Overall, bacterial community composition and diversity were affected primarily by time (gut microbiome maturation effect), whereas only minor impacts of the dietary Cu treatments were observed. Hence, none of the dietary Cu treatments influenced species richness (Chao1) or Shannon diversity index suggesting that ASV-level alpha diversity of the gut bacterial community was not affected by the dietary Cu treatments (Fig. S5AB). By contrast, the richness and diversity of the gut bacterial communities decreased as pigs aged (Kruskal-Wallis and Dunn's test, $P < 0.01$; Fig. S5C). Likewise, the bacterial community composition was highly affected by time (*perMANOVA*, $R^2 = 0.52$, $P < 0.001$; Figure 14) with only minor impacts of dietary Cu treatments. No clear overall impact of dietary treatments on the bacterial community composition was observed at the first time point (d 26; perMANOVA, $R^2 = 0.12$, $P = 0.224$; Fig. S6), but minor impacts were observed at the second time point as visualized in the NMDS ordination plot (d 116; *perMANOVA*, $R^2 = 0.19$, $P < 0.01$; Fig. S6).

Dietary Cu treatments altered the relative abundance of several genera (e.g. *Ligilactobacillus*, *Oscillospira*, *Prevotella*, *Roseburia*, *Schwartzia*, and *Streptococcus*) compared to the NC after 26 days (ANCOM-BC, $P < 0.05$; Fig. S7). However, only the relative abundance of *Dialister* and *Lactobacillus* was affected by the dietary treatments after 116 days (Fig. S7). The relative abundance of *Dialister* was higher in the 125 Cu_2O

group compared with the NC, whereas the relative abundance of *Lactobacillus* was higher in the NC group compared with 125 Cu_2O and 250 $CuSO_4$ or Cu_2O groups (d 116; ANCOM-BC, $P < 0.05$).

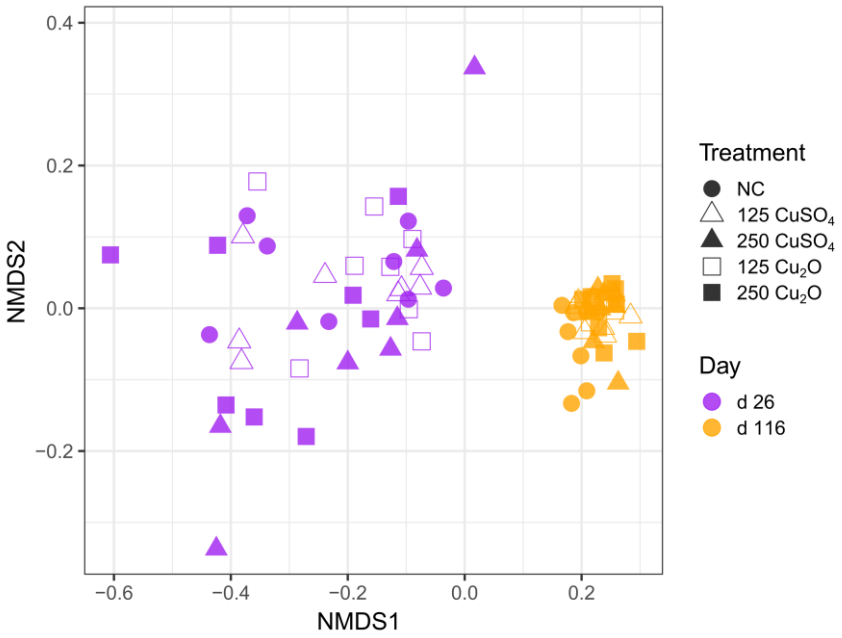


Figure 14. Differences in bacterial community composition as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity index across all fecal samples (n = 80) based on the relative abundance of amplicon sequence variants (ASVs). Communities grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}), and by time (day): 26 (purple dots), and 116 (orange dots).

We further explored the relative importance of different bacterial community assembly processes across different time points and treatments using iCAMP (Figure 15). Overall, drift was the most important community assembly mechanism across all time points and dietary Cu treatments. Moreover, dispersal limitation and homogenous selection also constituted major community assembly processes after 26 days, whereas homogenizing dispersal became important for shaping the bacterial community composition after 116 days. The importance of drift was highest within the abundant fraction of the community, whereas it was less important among taxa belonging to the

intermediate and rare fractions of the community. Drift, homogenous selection, and dispersal limitation were the most influential factors driving the changes in community assembly at the first time point (d 26) whereas at the second time point (d 116) drift was the most important process in community assembly of abundant taxa. Importantly, the different dietary Cu treatments had no clear impact on the relative importance of the different community assembly processes responsible for shaping the bacterial community.

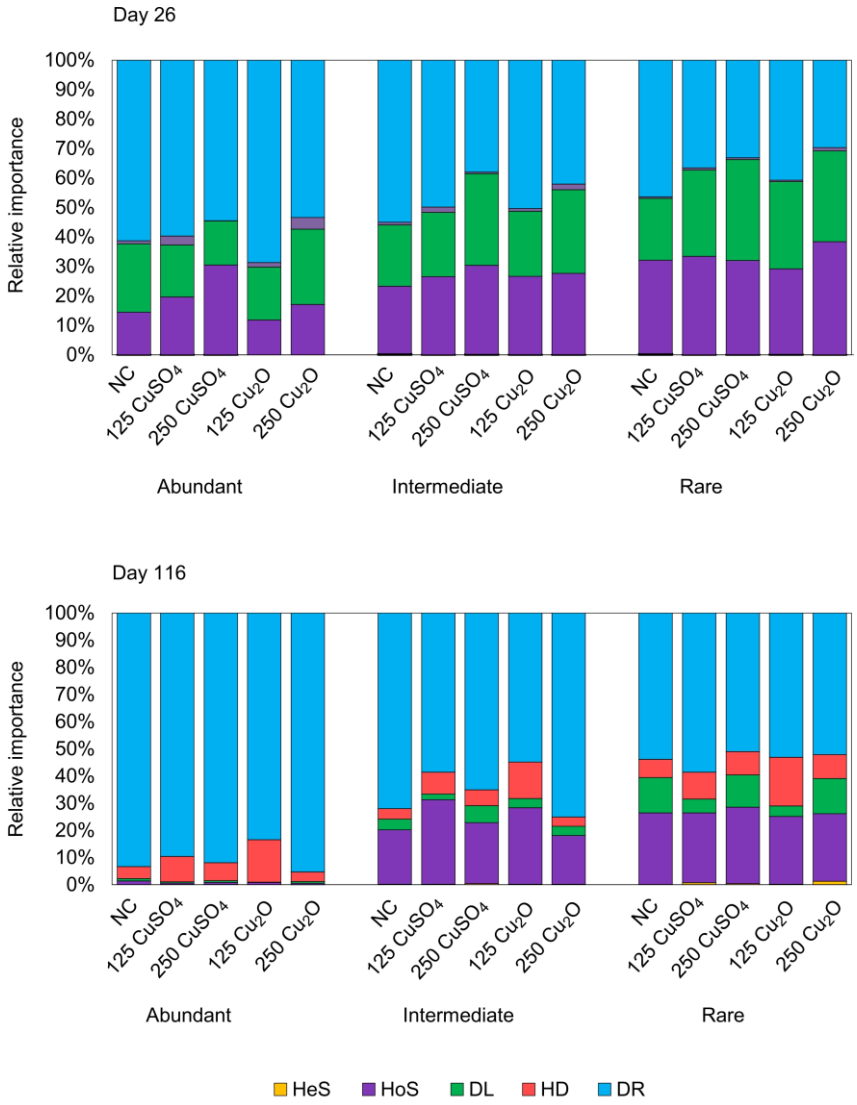


Figure 15. Relative importance of bacterial community assembly processes based on the principle of the null models employed by iCAMP in response to dietary Cu treatments: NC, 125 $CuSO_4$, 250 $CuSO_4$, 125 Cu_2O and 250 Cu_2O. The relative importance of heterogenous selection (HeS), homogenous selection (HoS), dispersal limitation (DL), homogenizing dispersal (HD), and drift (DR) was determined separately for abundant, intermediate, and rare bacterial taxa.

3.2. Impacts of high Cu diets on MRGs/MGEs of gut microbiota

The composition of the metal resistome was primarily affected by time (gut microbiome maturation effect; *perMANOVA*, $P < 0.001$; Figure 16), whereas it was not significantly impacted by dietary Cu at any of the time points (*perMANOVA*, $P > 0.1$; Fig. S8). Likewise, dietary Cu treatments did not markedly affect the number of detected MRGs (ANOVA, $P > 0.05$; Fig. S9A) or the relative abundance pattern of individual MRGs (Figure 17). The cumulative relative abundance of all quantified MRGs was also similar across the dietary Cu treatments after both 26 and 116 days (ANOVA, $P > 0.1$; Fig. S9B). The relative abundance of MRGs (normalized to the corresponding 16S rRNA gene copy number) ranged with an average of 0.20 across all samples at the first time point varying between 0.04 to 0.7, while at the second time point, the cumulative relative abundance ranged between 0.01 and 0.18 with an average of 0.07.

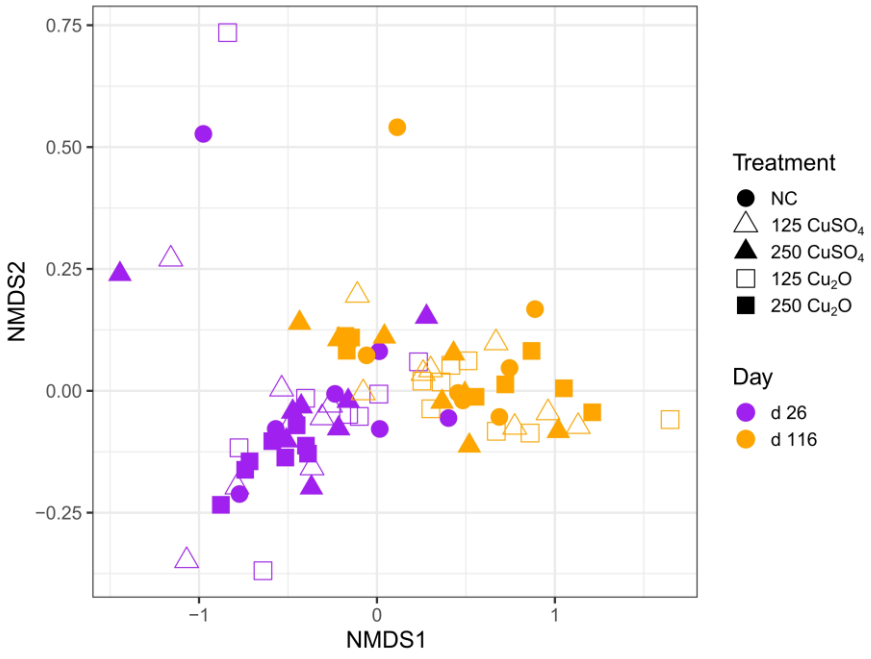


Figure 16. Differences in the metal resistance genes (MRGs) composition of the gut microbiome as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity index across all fecal samples ($n = 80$) grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}), and by time (day): 26 (purple dots), and 116 (orange dots).

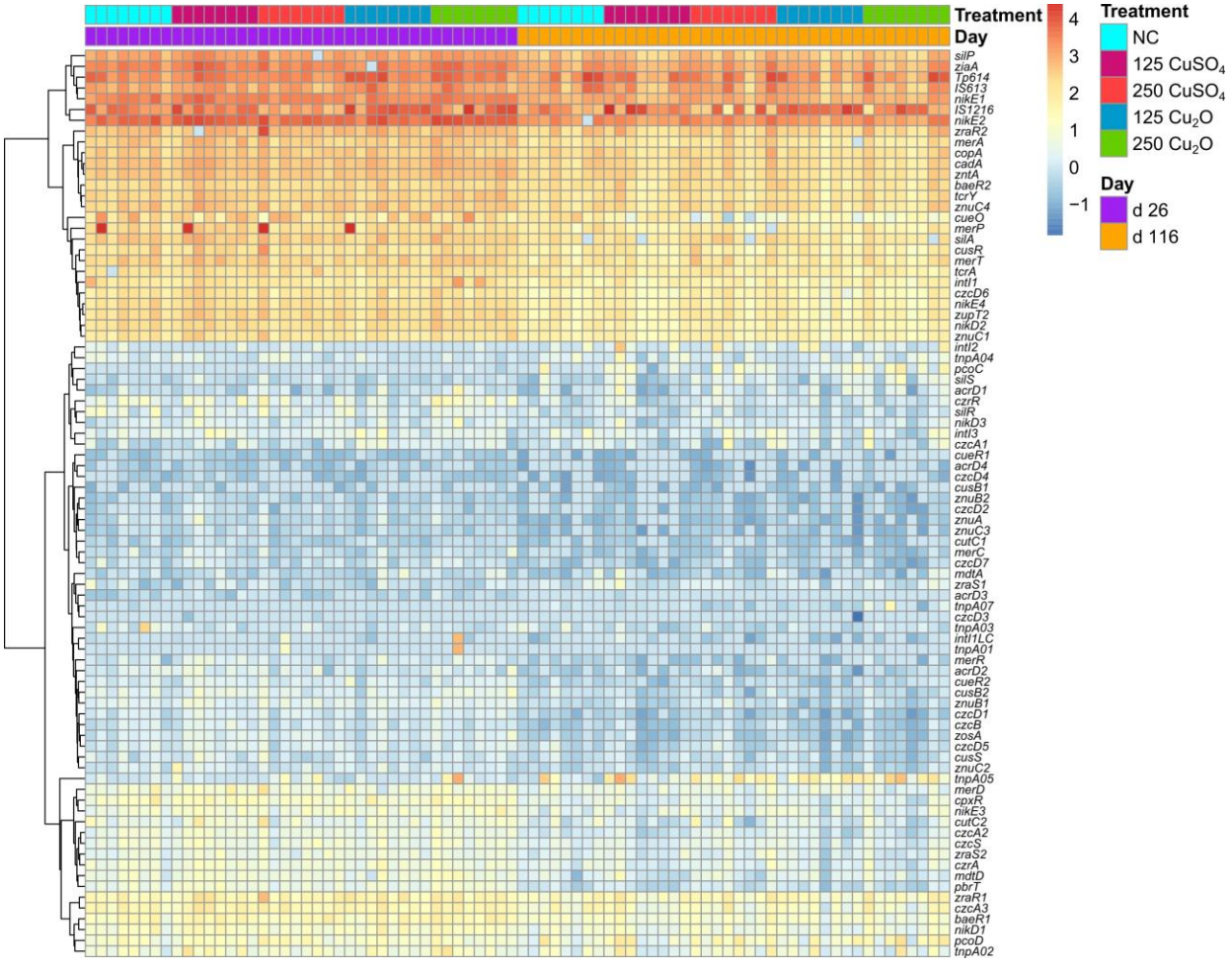


Figure 17. Heatmap showing the impacts of dietary Cu treatments and time on 86 gene targets related to metal resistance, mobile genetic elements, and multidrug resistance. The color gradient represents the log-transformed relative gene abundance (normalized to the corresponding 16S rRNA gene copy numbers). Rows represent the results of each primer set (assay) shown on the y-axis. Columns represent fecal samples (n = 80) grouped by dietary Cu treatments: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}) and by time (day): 26, and 116. Rows were clustered based on Euclidean distances.

By contrast, the dietary Cu treatments significantly affected the MGE profile (*perMANOVA*, $P < 0.05$; Fig. S10), although overall dietary Cu impacts were rather modest as indicated from the relative abundance pattern of individual MGEs (Figure 17). The dietary Cu treatments affected the cumulative relative abundance of transposases after 26 days (ANOVA, $P = 0.0002$; Fig. S9D), but not after 116 days (ANOVA, $P > 0.01$). Overall, the cumulative relative abundance (day 26 and 116; normalized to the

corresponding 16S rRNA gene copy number) of MGEs ranged from 0.02 and 0.3 with an average of 0.1. A more in-depth examination of individual MGEs revealed that dietary Cu treatments significantly impacted only the *IS1216* transposase gene after 26 days (ANOVA, $P = 0.016$; Fig. S11). Hence, the relative abundance of this gene was higher with Cu_2O (125 and 250 μg g^{-1}) supplementation as compared with 250 $CuSO_4$. Dietary Cu treatments did also not markedly affect the number of detected MGEs (ANOVA, $P > 0.05$; Fig. S9C).

The lacking or modest impacts of dietary Cu treatments on MRGs and MGEs were not surprising given that only a small subset of the gene targets have previously been indicated to confer resistance to Cu in bacteria. Hence, we specifically evaluated the impacts of dietary Cu on the relative abundance of 11 genes (*pcoC*, *copA*, *cusB*, *cutC*, *tcrA*, *cueO*, *cueR*, *cusR*, *cusS*, *tcrY*, and *pcoD*; targeted by 14 primers) previously shown to confer resistance to Cu (Figure 18). The number of detected CRGs was similar across all treatments (ANOVA, $P > 0.1$; Figure 18A).

Remarkably, dietary Cu treatments did not affect the cumulative relative abundance of CRGs (ANOVA, $P > 0.1$; Figure 18B), but the relative abundance of the *cueO* gene was decreased by dietary Cu treatments (only significant for 125 Cu_2O, 250 $CuSO_4$ and 250 Cu_2O treatments) relative to NC (ANOVA, $P < 0.001$; Figure 19). Moreover, the cumulative relative abundance of CRGs was significantly lower after 116 days as compared to 26 days (ANOVA, $P < 0.05$).

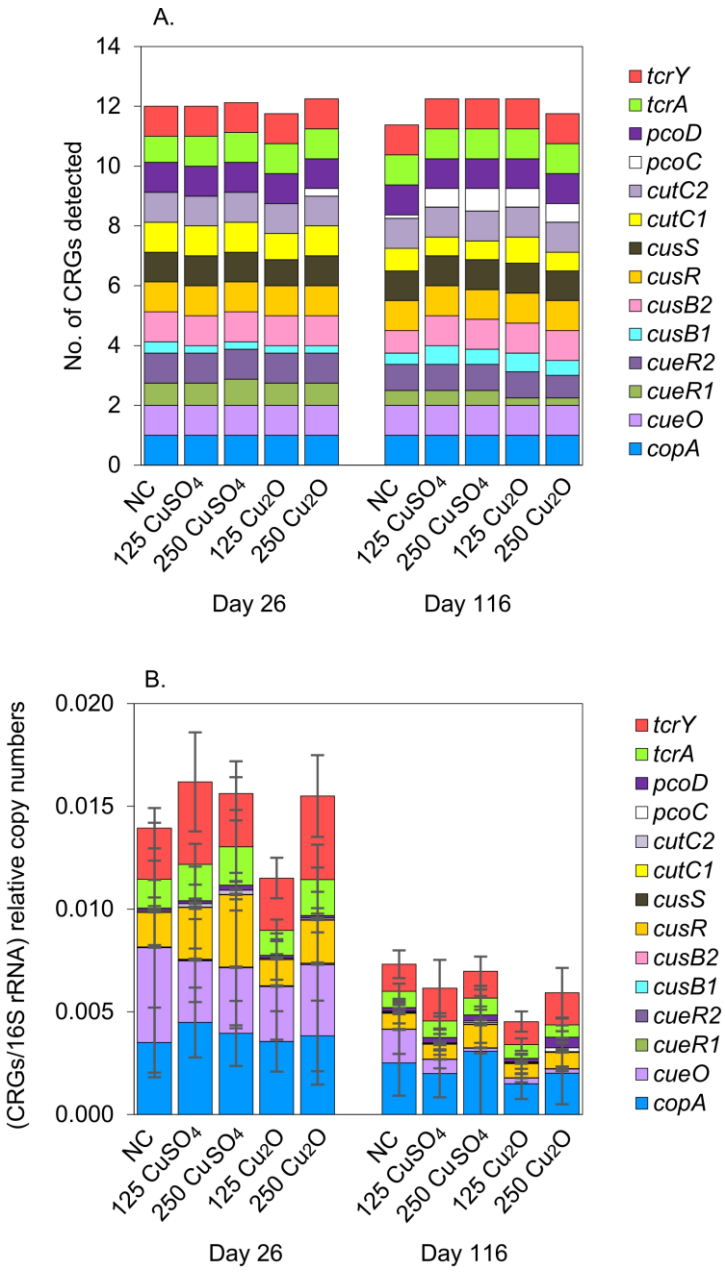


Figure 18. Impacts of dietary Cu treatments and time on the prevalence of Cu resistance genes (CRGs) in pig fecal samples (n = 80) grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}) and by time (day): 26, and 116. A. Average number of detected CRGs; B. Average cumulative relative abundance of CRGs. The cumulative relative abundance refers to the sum of relative abundance for all targeted CRGs. Error bars show the standard deviation.

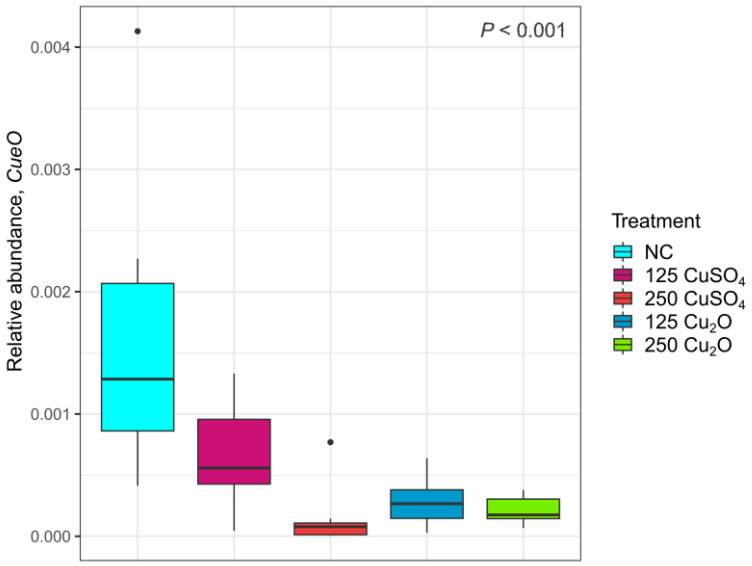


Figure 19. Boxplot showing the impacts of dietary Cu treatments on the average relative abundance of the *cueO* gene after 116 days of experiment. Relative gene abundance grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}). Boxes represent the interquartile range between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the boxes defines the median. Whiskers show the lowest and highest values within 1.5 times the IQR from the first and third quartiles. Outliers are represented by black dots. A one-way ANOVA test was used to evaluate differences in relative abundance between samples of different treatment groups (*P*-value is shown).

3.3. Linkage between bacterial community composition and MRGs/MGE profiles

Procrustes analysis was performed to investigate whether MRG or MGE profiles could be linked to bacterial community composition (i.e. ASVs present and their relative abundance). MRG profiles showed a significant correlation with bacterial community composition (Procrustes sum of squares M^2 = 0.54, R^2 = 0.68, *P* = 0.0001, 9999 free permutations; Fig. S12A), whereas there was no significant correlation between MGE profiles and the bacterial community composition (Procrustes sum of squares M^2 = 0.98, R^2 = 0.14, *P* = 0.33, 9999 free permutations; Fig. S12B). However, Spearman's rank correlation analysis revealed correlations (*P* < 0.05; Fig. S13) among some bacterial

genera and MRGs/MGEs within the swine gut microbiome. The most striking example was the co-occurrence of *Streptococcus* and the *IS1216* transposase gene with most entities being highly abundant in the two Cu_2O treatments after 26 days (R = 0.69, $P < 0.0001$; Figure 20). The abundance of *IS1216* transposase gene was also tightly correlated with *Streptococcus* after 116 days (R = 0.67, $P < 0.0001$), however, there was no significant difference between the treatments. The data from the second time point (day 116) revealed a remarkably tight correlation between the *cueO* gene and the genus *Lactobacillus* (R = 0.83, $P < 0.0001$; Figure 20). The correlation was also evident from the first time point (day 26) with no variation among the treatments (R = 0.92, $P < 0.0001$).

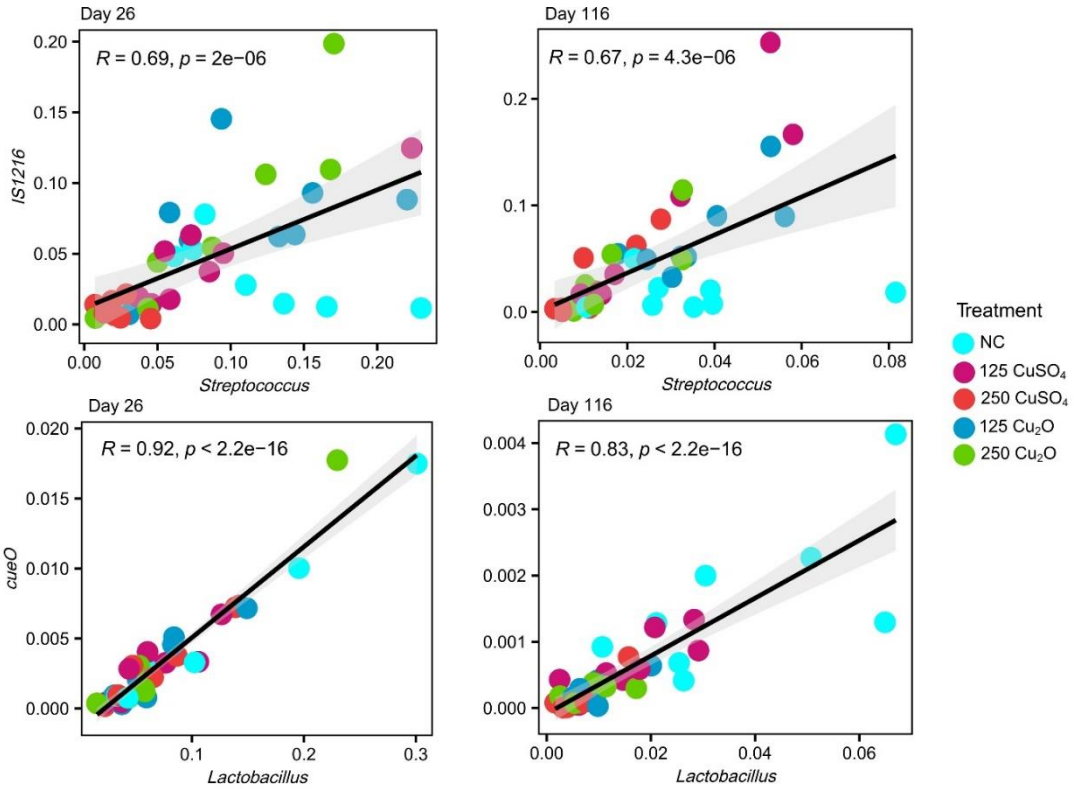


Figure 20. Spearman's rank correlations between the relative abundance of *IS1216* and *cueO* genes (genes/16S rRNA gene) and the relative abundance (%) of bacterial genera on day 26 or 116 of the experiment.

3.4. Phenotypic resistance to Cu and antibiotics in E. coli isolates

The negligible impacts of dietary Cu on metal resistance gene profiles (HT-qPCR) prompted us to further investigate phenotypic Cu resistance in gut bacterial isolates. *E. coli* was chosen as our indicator species as most of the strains enriched and isolated on the used non-selective medium belonged to this species. Supplementation with high levels of dietary Cu selected for Cu resistance in fecal *E. coli* isolates compared to the NC treatment (GLM, 250 $CuSO_4$, $P < 0.001$; 250 Cu_2O, $P < 0.05$; Figure 21A). Among the NC treatment ($n = 50$), only 24% were Cu resistant, whereas corresponding numbers for the 250 $CuSO_4$ ($n = 103$) and 250 Cu_2O ($n = 131$) treatments were 72% and 41%, respectively. The fraction of Cu-resistant *E. coli* isolates was significantly higher for the 250 $CuSO_4$ group as compared to the 250 Cu_2O group (GLM, $P < 0.05$).

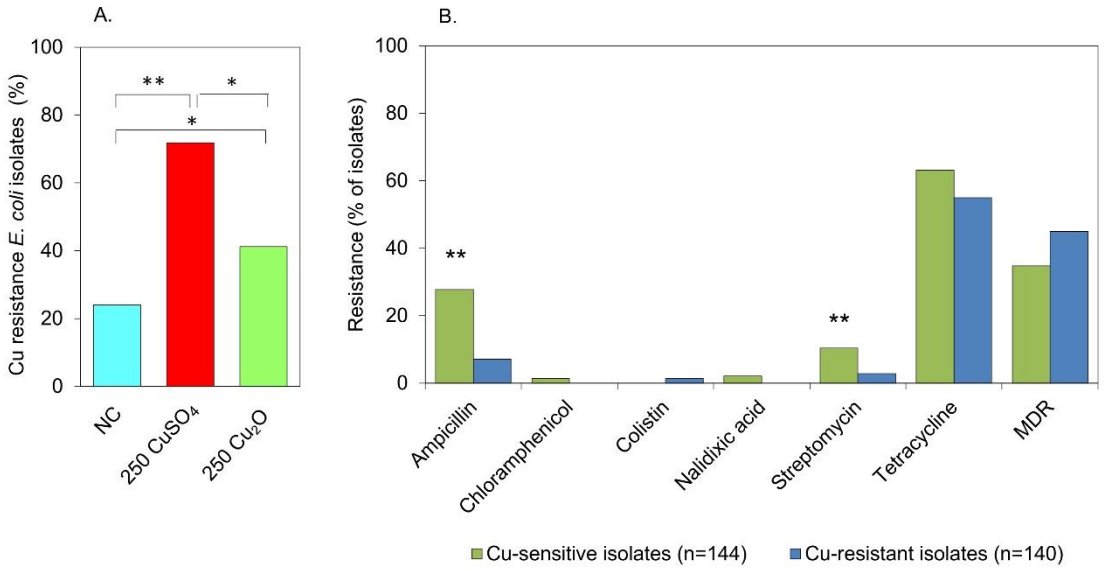


Figure 21. Phenotypic resistance to Cu and 6 antibiotics in fecal *Escherichia coli* (*E. coli*) isolates. A. Impacts of dietary Cu treatments on the frequency of Cu resistance among *E. coli* isolates (n = 284): NC (negative control; 50 isolates), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}; 103 isolates), and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}; 131 isolates); B. Frequency of antibiotic resistance and multidrug resistance (MDR) among Cu-sensitive (n = 144) and Cu-resistant (n = 140) *E. coli* isolates. The level of significance is indicated as follows: Generalized linear model (GLM); ** $P <$ 0.001, * $P < 0.05$.

Contrary to our hypothesis, Cu resistant *E. coli* isolates did not tend to be more resistant to antibiotics as compared to Cu susceptible isolates (Figure 21B). In fact, Cu resistant isolates were less resistant to ampicillin and streptomycin than corresponding Cu susceptible isolates (GLM, $P < 0.001$). A closer look at the results revealed that isolates resistant to ampicillin and streptomycin were mainly derived from the Cu sensitive isolates of the 250 Cu_2O group (GLM, $P < 0.001$; Fig. S14). Overall, supplementation of 250 Cu_2O, irrespective of Cu sensitivity, increased phenotypic resistance of *E. coli* isolates to ampicillin, and streptomycin compared with the NC and 250 $CuSO_4$ (GLM, $P < 0.05$; Fig. S15).

4. Discussion

4.1. Gut microbiome composition and bacterial community assembly processes

The gut microbiome was dominated by ASVs belonging to the phylum *Firmicutes* across all treatments and time points, which is in line with previous studies of the swine gut (Forouzandeh et al., 2022; Lamendella et al., 2011; Niu et al., 2015). However, the relative abundance of *Firmicutes* was 25 to 50% higher than in our previous study of the same feeding trial even though some of the samples used for DNA extraction were indeed taken from the same animals. This can be explained by the different DNA extraction kits used in the two studies following the commercial discontinuation of the kit used in the previous study (Brinck et al., 2023; Sperling et al., 2018). Nevertheless, the main conclusions regarding the impacts of dietary Cu on the gut bacterial resistome were similar in the two studies with a significant gut microbiome maturation effect (time) and rather minor impacts being observed for the dietary Cu treatments.

Dietary Cu treatments altered the relative abundance of a few genera. The relative abundance of *Lactobacillus* was reduced after 116 days in response to both dietary Cu treatments in line with our previous study (Brinck et al., 2023). The lacking effect of Cu supplementation on bacterial diversity and composition and the analogous effect of Cu supplements on swine gut microbiome in our experiment was in accordance with former studies where they compared the effect of 150 and 250 μg g^{-1} of Cu_2O and $CuSO_4$ on the composition of the microbial community in broilers ileum (Forouzandeh et al., 2021) or swine colon (Forouzandeh et al., 2022).

We investigated the relative importance of contrasting bacterial community assembly processes using iCAMP. No clear impacts of dietary Cu treatments on the relative importance of the community assembly processes responsible for shaping the bacterial community were observed. Drift was the most influential community assembly mechanism in the outcome of this experiment regardless of whether bacterial communities were stressed by high Cu concentration or not. Counterintuitively, drift became more important with pig age, even though it is generally thought that stochastic community assembly processes are most important during early phases of gut colonization (Seki et al., 2022). Our interpretation of the iCAMP output is that drift became more important over time for explaining animal-to-animal gut microbiome differences due to the observed convergence of bacterial community composition over time (this study, Brinck et al., 2023). Hence, the very small differences observed after 116 days were almost entirely driven by stochastic events.

4.2. Selection and co-selection of bacterial resistance to Cu and other metals

Cu supplementation had a negligible effect on the swine gut bacterial metal resistome, which was primarily affected by gut microbiome maturation (i.e. time). The absence of dietary Cu treatment effects on CRG and MRG profiles contrasts with our phenotypic Cu resistance data demonstrating a significantly higher frequency of Cu-resistant *E. coli* isolates from the two high-Cu dietary treatments than from the corresponding control treatment. The higher frequency of Cu-resistant isolates among the dietary Cu treatment groups indicates that elevated doses of dietary Cu actually had the ability to select for Cu resistant bacterial strains. This is in line with previous cultivation-dependent studies demonstrating that high doses of dietary Cu (125 to 208 μg Cu^{2+} g^{-1}) could be linked to acquired Cu resistance in *Enterococcus faecium* and *E. faecalis* via the plasmid-borne Cu resistance gene *tcrB* (Amachawadi et al., 2010, 2011; Hasman et al., 2006). Interestingly, more recent studies did not obtain evidence for an effect of high Cu supplementation on Cu resistance in *E. faecalis* or *E. coli* isolates collected from broilers feces (Forouzandeh et al., 2021) or in *E. faecalis* isolates collected from swine feces (Capps et al., 2020; Villagómez-Estrada et al., 2020).

Under the influence of dietary Cu treatments, only the relative abundance of the *CueO* gene was affected, but the Cu supplemented diet actually decreased *CueO* abundance. *CueO* encodes for an enzyme with a high cuprous oxidase activity, which protects *E. coli* against Cu toxicity, but the CueO enzyme may also have other cell functions unrelated to Cu (Singh et al., 2004). Altogether, the HT-qPCR MRG chip data did thus not provide any evidence for selection of CRGs even though dietary Cu selected for phenotypic Cu resistance in *E. coli* isolates. This finding demonstrates that the relative abundances of CRGs targeted by the MRG chip may constitute a poor proxy for

phenotypic Cu resistance in specific groups of bacteria and it is thus advised to complement the MRG chip with phenotypic resistance data for specific species of bacteria to infer whether high levels of dietary Cu can select for Cu resistance via toxicant-induced succession. In addition, it should also be mentioned that some CRGs (e.g. *pcoA*, *cusA*, and *tcrB*) were not covered by the used MRG chip.

Rather than indicating an important role of dietary Cu in shaping the MRG profiles, our results showed a significant correlation between MRG profiles and bacterial community composition. This is consistent with our previous study showing a similar correlation between ARG profiles and bacterial community composition (Brinck et al., 2023). Although we did not observe a major treatment effect on the overall gut microbiome, the relative abundance of *Lactobacillus* declined in response to Cu treatments after 116 days. Previous studies have demonstrated a similar decrease in *Lactobacillus* abundance following Cu or Zn supplementation in pigs and poultry (Forouzandeh et al., 2021; Højberg et al., 2005; Mei et al., 2009). Likewise, the relative abundance of *cueO* decreased under the influence of elevated Cu. These findings exemplify how dietary Cu treatments may affect the composition of the swine gut bacterial metal resistome via changes in bacterial community composition.

4.3. Cu-induced co-selection of MGEs

Unlike MRGs, the MGE profiles were in general not well correlated to changes in the bacterial community composition, but the dietary Cu treatments did affect the MGE profile to some extent. Specifically, the *IS1216* transposase gene was significantly more abundant in dietary treatments with Cu_2O (125 and 250 µg g^{-1}) suggesting that this Cu source could possibly imply an increased risk for horizontal transfer of antibiotic

resistance determinants among gut bacteria. The abundance of the *IS1216* transposase gene was highly correlated with *Streptococcus* after both 26 and 116 days, but *IS1216* may also play a prominent role for gene transfer in *Enterococcus* species of animal origin (Liu et al., 2012; Xu et al., 2010).

4.4. Cu-induced co-selection of antibiotic resistance in pigs

Pig production is generally considered a hotspot for the development and transfer of antibiotic resistance and since metals such as Cu and Zn are frequently used in parallel with antibiotics it is often also considered a hotspot specifically for metal-induced co-selection of antibiotic resistance (Zhao et al., 2021). Indeed, it has been shown that multi-resistance plasmids conferring resistance to both metals and antibiotics are present in gut bacteria and that they are probably enriched in bacteria of livestock fecal origin compared with other environments (Fang et al., 2016; Pál et al., 2015). It has also been shown that exposure to Cu and Zn can co-select for increased levels of resistance against β-lactams in *E. coli* isolates from pigs in Germany (Hölzel et al., 2012) and against chloramphenicol and ciprofloxacin in *E. coli* isolates from pigs in China (Zhang et al., 2019). Based on our phenotypic resistance data from *E. coli* isolates from pigs in Illinois, we could reject the hypothesis that Cu resistant *E. coli* isolates were more resistant to contrasting classes of antibiotics than corresponding Cu susceptible isolates were. Hence, our data did not indicate co-selection of Cu resistance and antibiotic resistance in *E.coli* from pigs supplemented with dietary Cu supplements in accordance with our previous microbiome-wide study showing that the studied dietary Cu treatments did not co-select for ARGs (Brinck et al., 2023).

5. Conclusion

In conclusion, we propose that the lacking evidence for Cu-induced co-selection of antibiotic resistance in our pig feeding trial (Brinck et al., 2023; this study) can be explained at least in part by the negligible impacts of the imposed dietary Cu treatments on the gut bacterial metal resistome as observed in this study. Hence, dietary Cu did not select for known CRGs or multidrug resistance genes targeted by the used HT-qPCR MRG Chip indicating a low risk for co-selection via co-resistance or cross-resistance mechanisms, respectively (Baker-Austin et al., 2006). We hypothesize that the negligible impacts of dietary Cu on the gut microbiome reflect the fact that high levels of dietary Cu have been in use for many pig generations and associated legacy effects of this agricultural practice. Hence, CRGs may already have become stably integrated into the faecal microbiome consistent with the weaker impacts of dietary Cu on Enterococci in recent studies (Capps et al., 2020; Villagómez-Estrada et al., 2020) as compared to older studies (Amachawadi et al., 2010, 2011; Hasman et al., 2006). Similar legacy effects have previously been proposed for feeding trials with elevated doses of antibiotics in pigs showing no development of antibiotic resistance (Pollock et al., 2020) and may have important consequences such as enhanced persistence of resistance to antimicrobial agents even at low concentrations (Salyers & Amábile-Cuevas, 1997; Andersson & Hughes, 2011).

Despite the growing knowledge on the antimicrobial activity of Cu, much remains to be learned about the impacts of dietary Cu supplements on the swine gut bacterial resistome and its implications for transmission and dissemination of antibiotic resistance to humans. Cu-based feed additives can have significant growth-promoting effects in pigs, but clearly this benefit must be carefully balanced against the potential

risks for co-selection and persistence of antibiotic resistance in pig gut bacteria and the subsequent transfer of antibiotic resistance to humans via various pathways (Zhao et al., 2021).

6. Supplementary tables and figures

Table S1. Summary of primers (*n* = 87), gene targets (*n* = 56), and their related functions on the MRG chip and mobile genetic elements. Complete information about the designed primer sequences can be found in the original study (Zhu et al., 2022)

Metal type	Primer	Gene	Gene function
Ag	*silA1*	*silA*	Efflux
	silP1	*silP*	Efflux
	silR1	*silR*	Regulator
	silS1	*silS*	Regulator
Cu	*pcoC*	*pcoC*	Binding
	copA1	*copA*	Efflux
	copB1	*copB*	Efflux
	cusB1, cusB2	*cusB*	Efflux
	cutC1, cutC2	*cutC*	Efflux
	tcrA1	*tcrA*	Efflux
	cueO1	*cueO*	Redox
	cueR1, cueR2	*cueR*	Regulator
	cusR1	*cusR*	Regulator
	cusS1	*cusS*	Regulator
	tcrY1	*tcrY*	Regulator
	pcoD	*pcoD*	Uptake
Hg	*merP*	*merP*	Binding
	merB	*merB*	Cleave
	merA	*merA*	Redox
	merD	*merD*	Regulator
	merR	*merR*	Regulator
	merC	*merC*	Uptake
	merT	*merT*	Uptake
Ni	*nikD1, nikD2, nikD3*	*nikD*	Uptake
	nikE1, nikE2, nikE3, nikE4	*nikE*	Uptake
Zn	*ziaA1*	*ziaA*	Efflux
	zraR1, zraR2	*zraR*	Regulator
	zraS1, zraS2	*zraS*	Regulator
	znuA1	*znuA*	Uptake
	znuB1, znuB2	*znuB*	Uptake
	znuC1, znuC2, znuC3, znuC4	*znuC*	Uptake
	zosA1	*zosA*	Uptake
	zupT2	*zupT*	Uptake
Zn/Cd/Co/Pb	*cadA1*	*cadA*	Efflux
	czcA1, czcA2, czcA3	*czcA*	Efflux
	czcB1	*czcB*	Efflux
	czcC1	*czcC*	Efflux
	czcD1, czcD2, czcD3, czcD4, czcD5, czcD6, czcD7	*czcD*	Efflux
	zntA1	*zntA*	Efflux

	czcS1	*czcS*	Regulator
	czrA1	*czrA*	Regulator
	czrR1	*czrR*	Regulator
	pbrT1	*pbrT*	Uptake
Multidrug and metal resistance	*acrD1, acrD2, acrD3, acrD4*	*acrD*	Efflux
	mdtA1	*mdtA*	Efflux
	mdtD1	*mdtD*	Efflux
	baeR1, baeR2	*baeR*	Regulator
	cpxR1	*cpxR*	Regulator
Mobile genetic elements	*intI1(clinic)*	*intI1(clinic)*	integrase
	intI1LC	*intI1LC*	integrase
	intI2	*intI2*	integrase
	intI3	*intI3*	integrase
	IS1216	*IS1216*	transposase
	IS613	*IS613*	transposase
	tnpA1, tnpA2, tnpA3, tnpA4, tnpA5, tnpA7	*tnpA*	transposase
	Tp614	*Tp614*	transposase

Table S2. Identified isolates (*n* = 95) by Sanger sequencing using the 27F 16S rRNA gene primer after growing bacteria on R2A medium. Bacteria were extracted from pig fecal samples (*n* = 24) derived from the different treatment groups: NC (negative control), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}). Strains were identified using BLAST ® optimized for highly similar sequences

Sample No.	Treatment	Sequence Length (bp)	Strain	Percent Identity
1	NC	1342	Aerococcus viridans	99.57%
2	NC	1418	Arthrobacter sp.	99.72%
3	NC	1408	Bacillus acidicola	99.71%
4	NC	1183	Bacillus altitudinis	99.79%
5	250 Cu_2O	1347	Bacillus cereus	99.26%
6	NC	1342	Bacillus halotolerans	99.25%
7	250 $CuSO_4$	1354	Bacillus halotolerans	99.17%
8	NC	1362	Bacillus pumilus	99.25%
9	NC	1351	Bacillus sp. HNI91	99.17%
10	NC	1400	Bacillus subtilis	99.72%
11	250 Cu_2O	1387	Bacillus subtilis	99.83%
12	250 Cu_2O	1398	Bacillus subtilis	99.73%
13	250 $CuSO_4$	1399	Bacillus wiedmannii	99.29%
14	NC	158	Bacterium NLAE-zl-H119	98.08%
15	250 $CuSO_4$	1329	Bacterium Streptomyces diastaticus	100.00%
16	NC	1346	Brevibacillus sp. sjf_26	99.83%
17	NC	1631	Corynebacterium sp.	99.02%
18	NC	1327	Corynebacterium sp.	99.53%
19	NC	1410	*E. coli*	99.09%
20	NC	1350	*E. coli*	99.00%
21	NC	1398	*E. coli*	97.14%
22	NC	1383	*E. coli*	98.93%
23	NC	1419	*E. coli*	99.81%
24	NC	1421	*E. coli*	98.85%
25	NC	1352	*E. coli*	99.09%
26	NC	1352	*E. coli*	99.18%
27	NC	1352	*E. coli*	99.01%
28	NC	1379	*E. coli*	98.05%
29	250 $CuSO_4$	1376	*E. coli*	98.93%
30	250 $CuSO_4$	1391	*E. coli*	98.93%
31	250 $CuSO_4$	1437	*E. coli*	98.93%
32	250 $CuSO_4$	1418	*E. coli*	98.92%
33	250 $CuSO_4$	1452	*E. coli*	98.93%
34	250 $CuSO_4$	1407	*E. coli*	98.93%
35	250 $CuSO_4$	1384	*E. coli*	99.01%
36	250 $CuSO_4$	1408	*E. coli*	99.72%
37	250 $CuSO_4$	1419	*E. coli*	99.18%
38	250 $CuSO_4$	1445	*E. coli*	99.01%
39	250 $CuSO_4$	1419	*E. coli*	99.18%
40	250 $CuSO_4$	1381	*E. coli*	98.61%

41	250 $CuSO_4$	1354	*E. coli*	99.17%
42	250 $CuSO_4$	1380	*E. coli*	99.09%
43	250 $CuSO_4$	1460	*E. coli*	98.10%
44	250 $CuSO_4$	1472	*E. coli*	99.81%
45	250 $CuSO_4$	1365	*E. coli*	98.99%
46	250 $CuSO_4$	1384	*E. coli*	98.41%
47	250 $CuSO_4$	1426	*E. coli*	98.76%
48	250 $CuSO_4$	1369	*E. coli*	98.93%
49	250 Cu_2O	1352	*E. coli*	99.01%
50	250 Cu_2O	1313	*E. coli*	99.83%
51	250 Cu_2O	1402	*E. coli*	99.01%
52	250 Cu_2O	1426	*E. coli*	98.54%
53	250 Cu_2O	1418	*E. coli*	99.03%
54	250 Cu_2O	1428	*E. coli*	99.01%
55	250 Cu_2O	1440	*E. coli*	99.09%
56	250 Cu_2O	1420	*E. coli*	99.01%
57	250 Cu_2O	1346	*E. coli*	99.18%
58	250 Cu_2O	1380	*E. coli*	98.93%
59	250 Cu_2O	1435	*E. coli*	99.72%
60	250 Cu_2O	1456	*E. coli*	99.01%
61	250 Cu_2O	1417	*E. coli*	99.18%
62	250 Cu_2O	1398	*E. coli*	98.78%
63	250 Cu_2O	1356	*E. coli*	98.51%
64	250 Cu_2O	1446	*E. coli*	99.10%
65	250 Cu_2O	1456	*E. coli*	98.85%
66	250 Cu_2O	1419	*E. coli*	98.93%
67	250 Cu_2O	1415	*E. coli*	99.18%
68	250 Cu_2O	1432	*E. coli*	99.18%
69	250 Cu_2O	1317	*E. coli*	99.09%
70	250 Cu_2O	608	*E. coli*	98.09%
71	250 Cu_2O	1312	*E. coli*	99.01%
72	250 Cu_2O	1283	*E. coli*	98.17%
73	250 Cu_2O	1449	*E. coli*	98.98%
74	NC	1381	Empedobacter falsenii	99.26%
75	NC	157	Enterobacter hormaechei	99.01%
76	NC	164	Enterobacter sp.	100.00%
77	250 $CuSO_4$	271	Enterobacter sp.	100.00%
78	250 $CuSO_4$	223	Enterobacter sp.	100.00%
79	250 $CuSO_4$	474	Enterobacter sp.	100.00%
80	250 Cu_2O	156	Enterobacter sp.	100.00%
81	250 $CuSO_4$	172	Enterobacter sp.	100.00%
82	250 Cu_2O	150	Kosakonia oryzae	98.82%
83	250 Cu_2O	149	Kosakonia oryzae	100.00%
84	NC	1607	Lysinibacillus sp.	100.00%
85	NC	1305	Rothia halotolerans	98.51%
86	NC	1276	Rothia halotolerans	99.54%
87	250 $CuSO_4$	1373	Staphylococcus saprophyticus	99.43%
88	NC	1425	Staphylococcus sp.	98.32%
89	250 Cu_2O	1410	Streptomyces sp. S417	98.99%

90	NC	189	uncultured bacterium	100.00%
91	NC	462	Low CRL[a]	×
92	NC	565	Low CRL	×
93	250 $CuSO_4$	150	Low CRL	×
94	250 $CuSO_4$	234	Low CRL	×
95	250 $CuSO_4$	277	Low CRL	×

[a] CRL: Contiguous Read Length

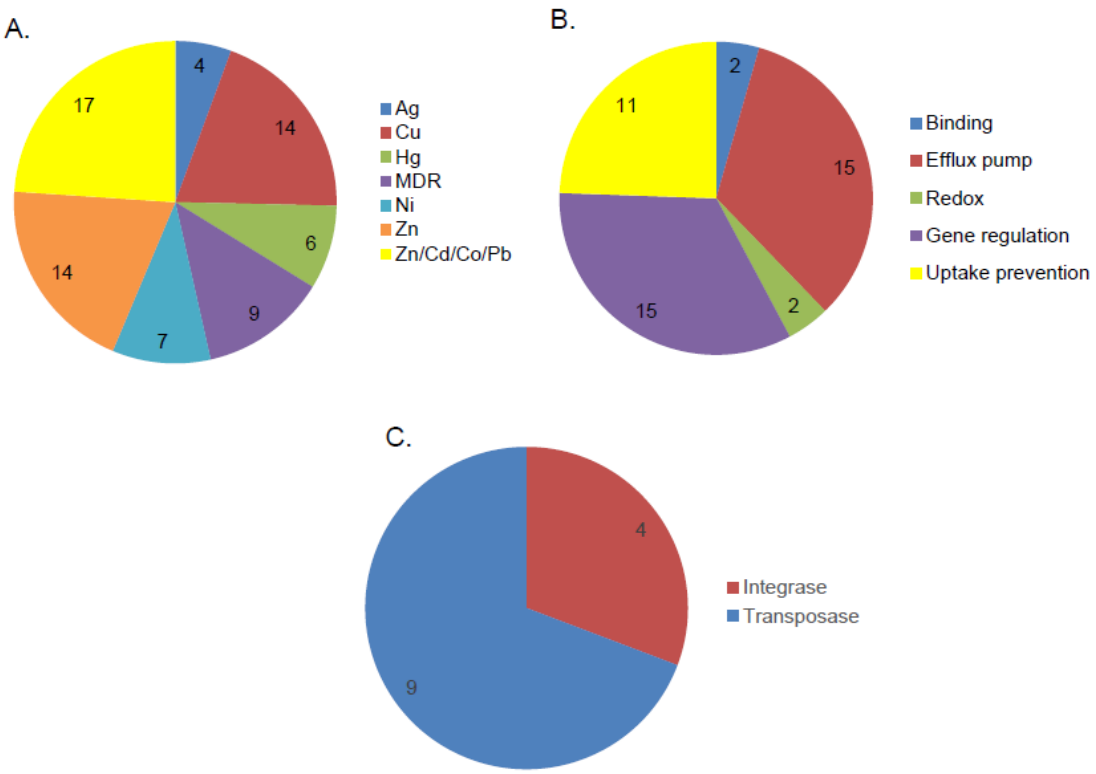


Fig. S1. Classification of primers (*n* = 84) targeting metal resistance genes (MRGs) and mobile genetic elements (MGEs) grouped according to: **A.** Metal to which the targeted gene confers resistance; **B.** Resistance mechanism; **C.** Mobile genetic element group. The numbers refer to the counts of targeted genes in each group. MDR: multidrug resistance genes.

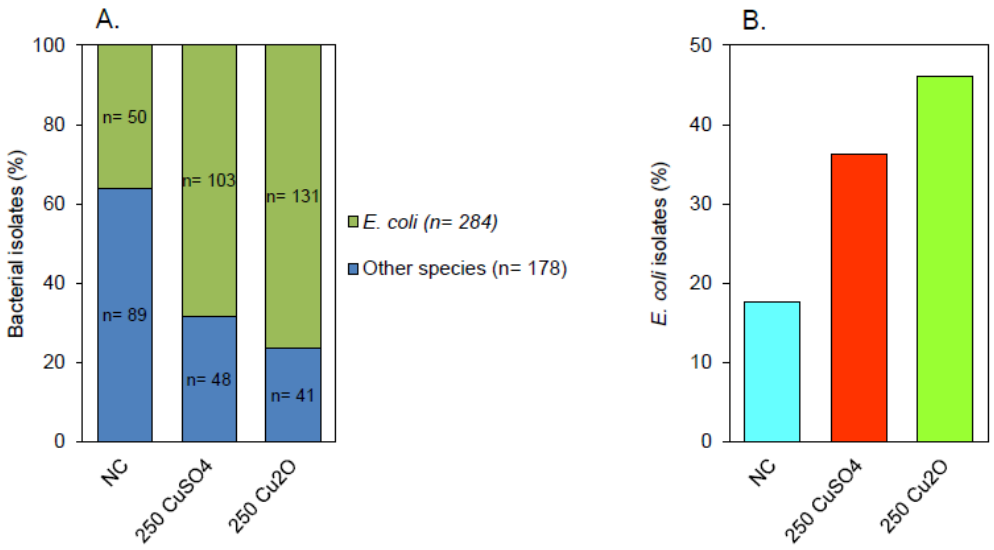
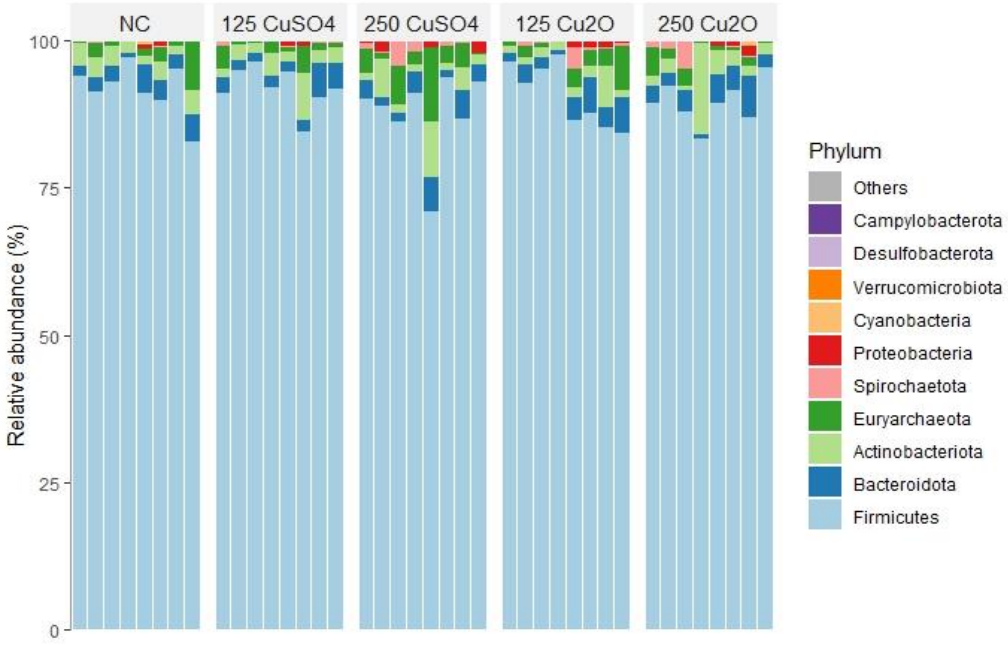


Fig. S2. Frequency and distribution of detected *E. coli,* based on *E. coli* selective medium, among fecal isolates (*n* = 462) from the different treatment groups: NC (negative control; 139 isolates), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}; 151 isolates), and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}; 172 isolates). **A.** Frequency of detected *E. coli*; **B.** Distribution of detected *E. coli* between treatment groups.

Day 26



Day 116

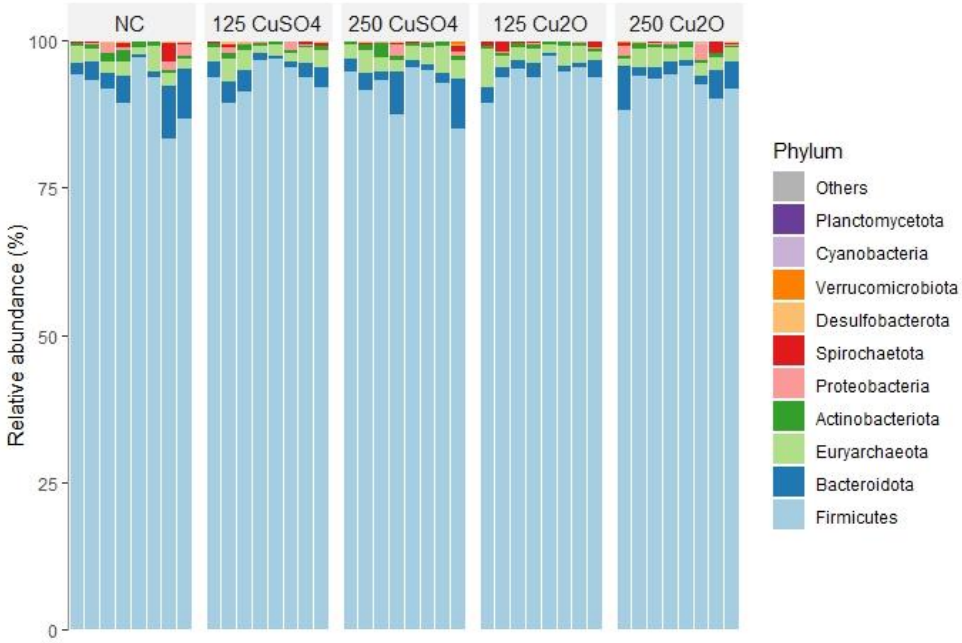
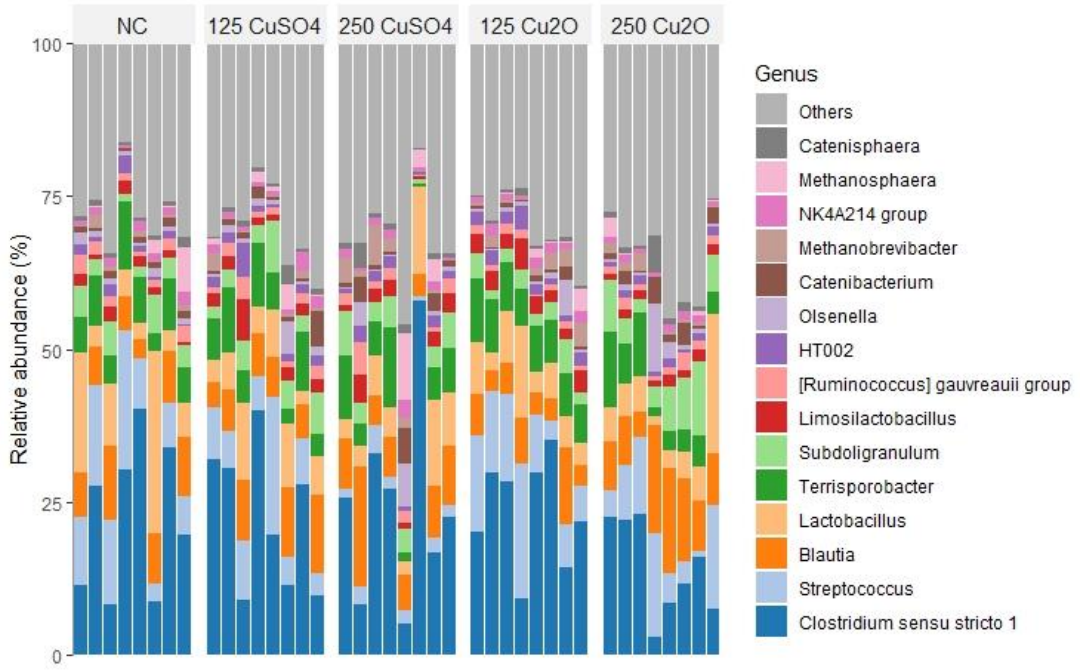


Fig. S3. Impacts of dietary Cu treatments and time (day 26 and 116) on the phylum-level bacterial community composition in individual swine fecal samples (*n* = 80). NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}).

Day 26



Day 116

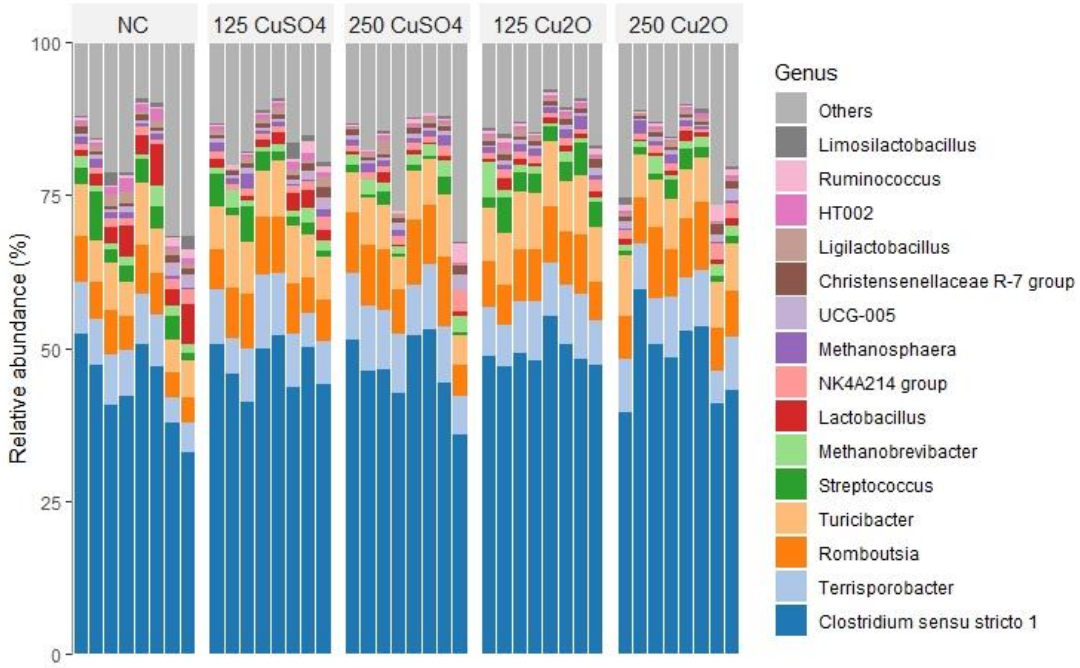


Fig. S4. Impacts of dietary Cu treatments and time (day 26 and 116) on the genus-level bacterial community composition in individual swine fecal samples (*n* = 80). NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}).

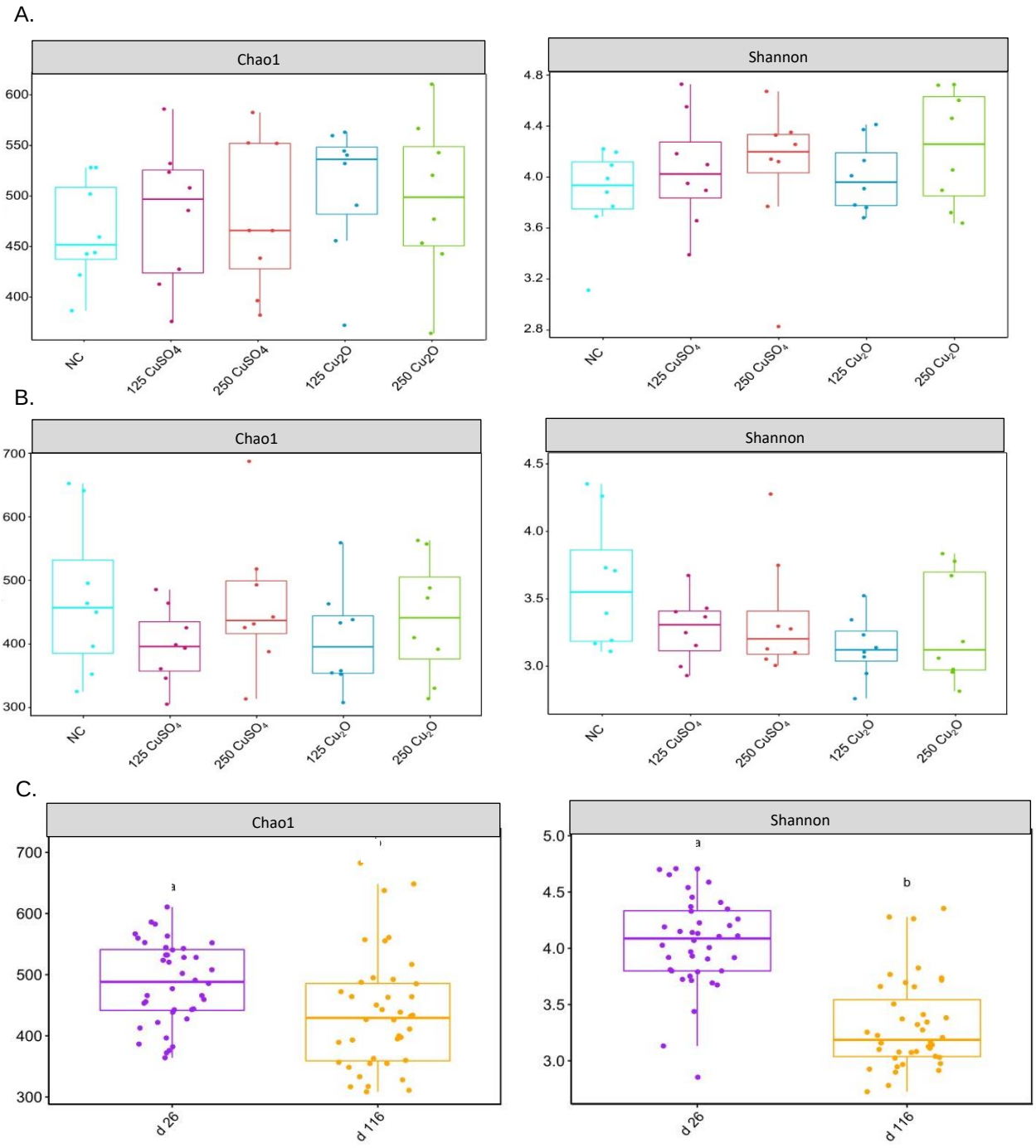


Fig. S5. Boxplots showing alpha diversity (Chao1 and Shannon Index) of the bacterial community across all fecal samples (*n* = 80). Communities grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}), during gut microbiome maturation (day): **A.** 26, **B.** 116, and **C.** All data (26 and 116 days). Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the boxes defines the median. Every sample is represented by a colored dot. Dots beyond the end of the whiskers are outliers. Solid lines and asterisks indicate a significant difference between groups.

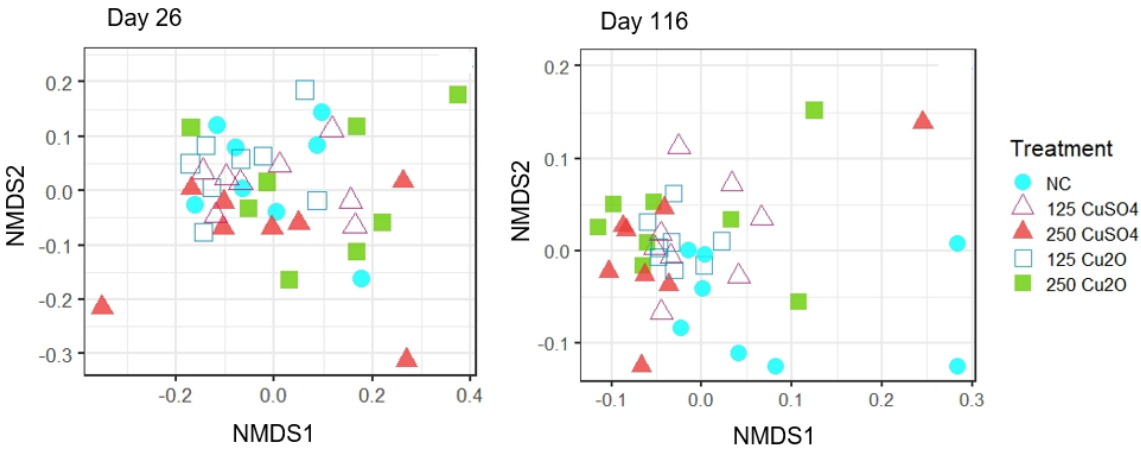


Fig. S6. Impacts of dietary Cu treatments and time (day 26 and 116) on bacterial community composition as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity metrics based on 16S rRNA gene amplicon sequence data across all fecal samples (*n* = 80). NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}).

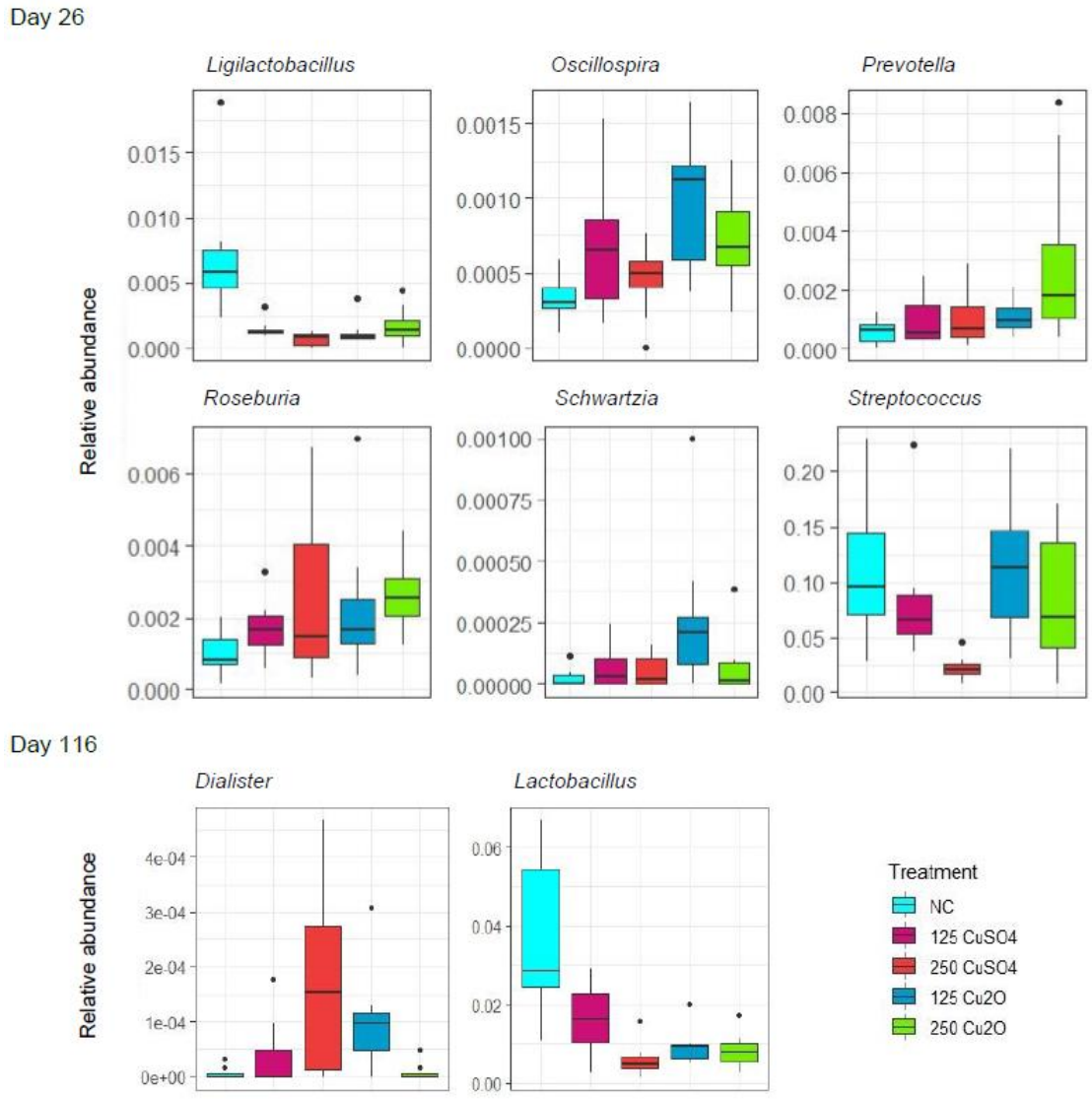


Fig. S7. Boxplots showing the genera that were significantly impacted by at least one of the dietary Cu treatments either on day 26 or 116 as identified by ANCOM-BC. NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}). Boxes represent the interquartile range between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the boxes defines the median. Whiskers show the lowest and highest values within 1.5 times the IQR from the first and third quartiles. Outliers are represented by black dots.

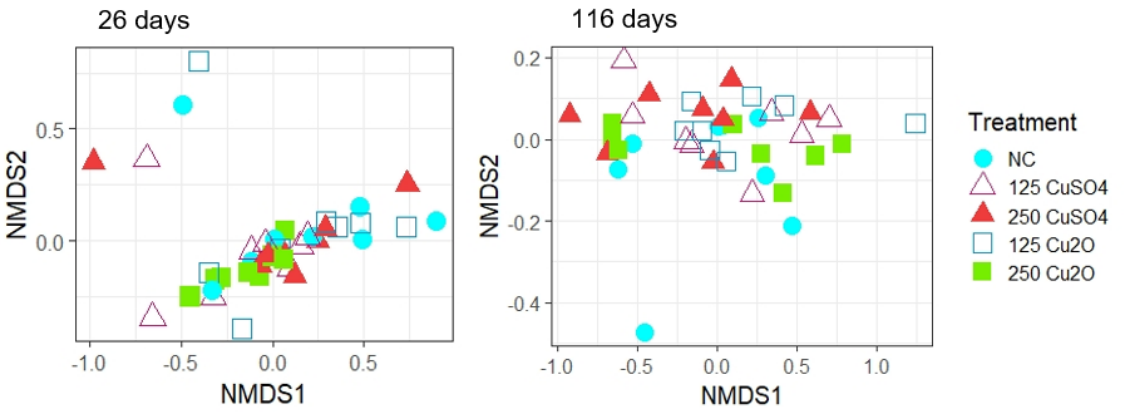


Fig. S8. Impacts of dietary Cu treatments and time (day 26 and 116) on metal resistance gene (MRG) profiles as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity metrics across all pig fecal samples (*n* = 80). NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}), and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}).

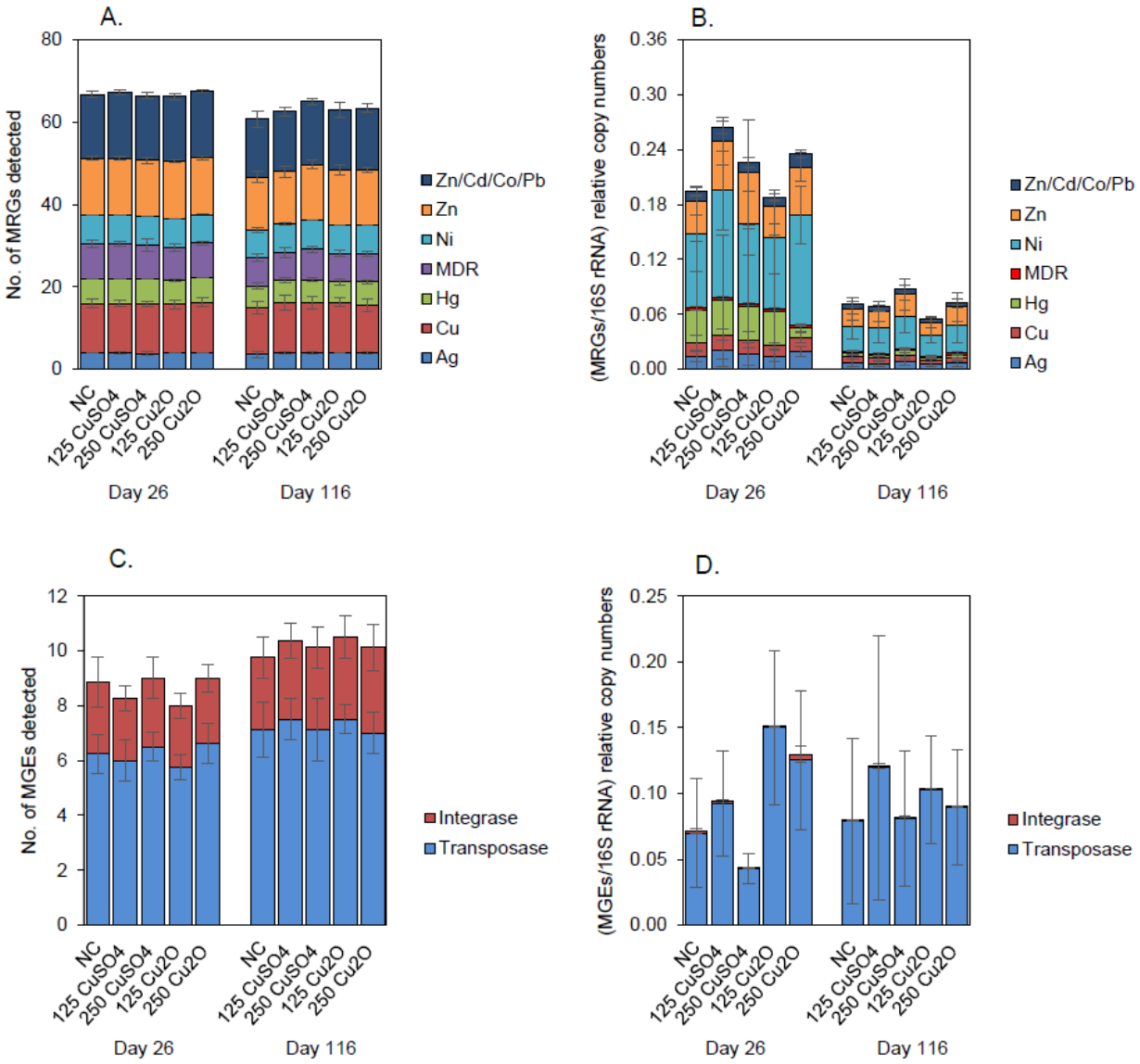


Fig. S9. Impacts of dietary Cu treatments on metal resistance genes (MRGs) and mobile genetic elements (MGEs) in pig fecal samples (*n* = 80) grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 μg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 μg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 μg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 μg g^{-1}) and by time (day): 26, and 116. **A.** Average number of unique MRGs; **B.** Average cumulative relative abundance of MRGs conferring resistance to different metals or groups of metals; **C.** Average number of unique MGEs; D. Average cumulative relative abundance of MGEs. The cumulative relative abundance refers to the sum of relative abundance for all targeted MRGs or MGEs, respectively. Error bars show the standard deviation.

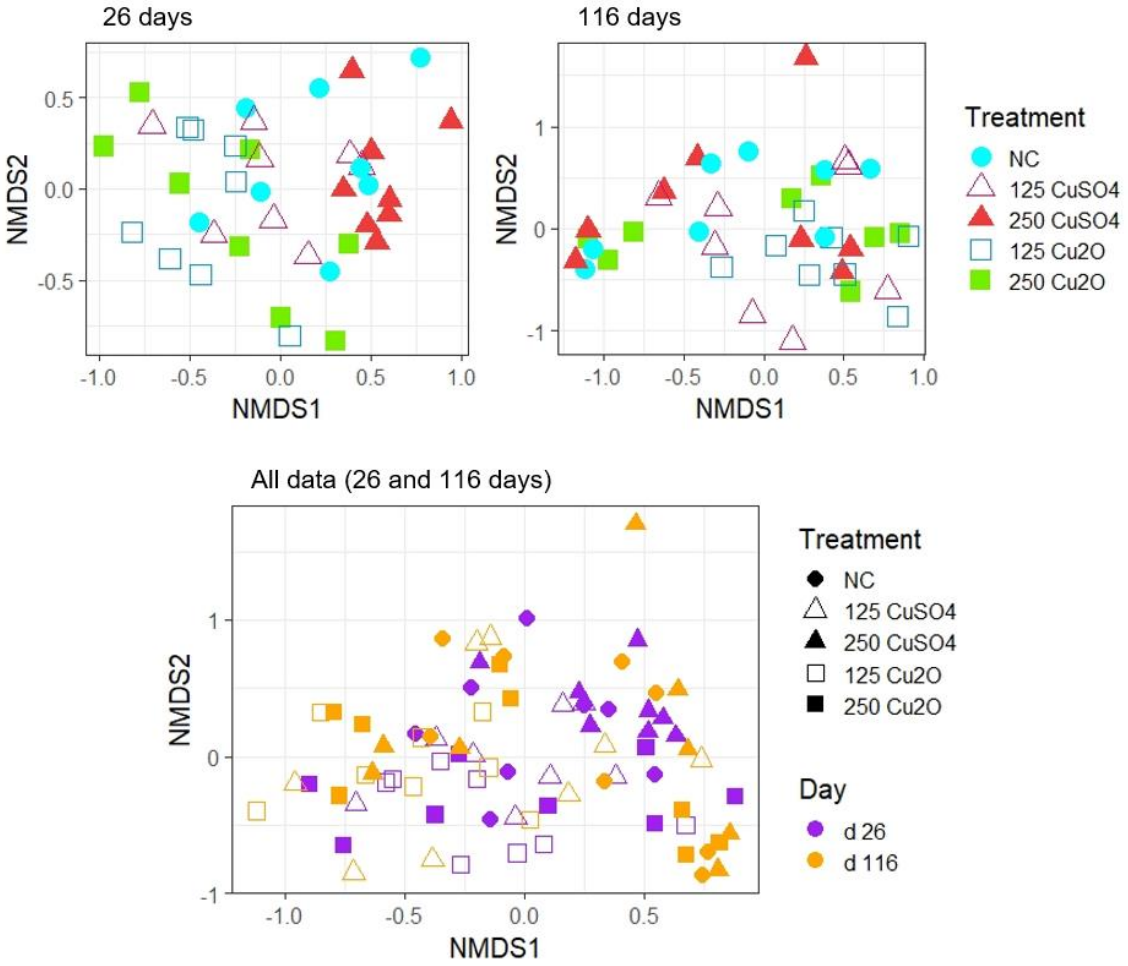


Fig. S10. Impacts of dietary Cu treatments and time (day 26 and 116) on mobile genetic element (MGE) profiles as revealed by non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis dissimilarity metrics across all pig fecal samples (*n* = 80). NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}).

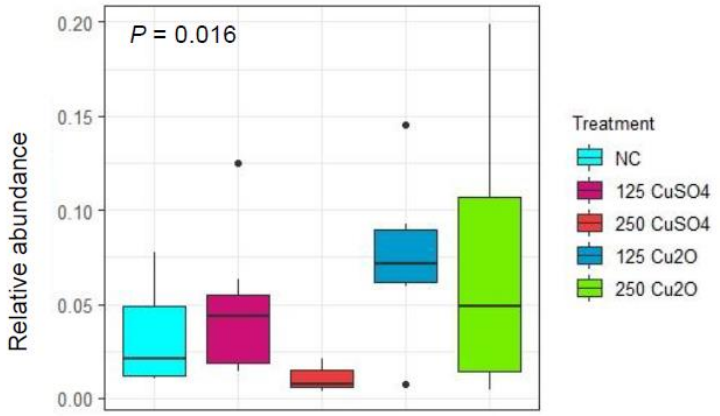


Fig. S11. Boxplots showing the impacts of dietary Cu treatments on the average relative abundance of the *IS1216* transposase gene after 26 days of the experiment. Relative gene abundance grouped by treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}). Boxes represent the interquartile range between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the boxes defines the median. Whiskers show the lowest and highest values within 1.5 times the IQR from the first and third quartiles. Outliers are represented by black dots. A one-way ANOVA test was used to evaluate differences in relative abundance between samples of different treatment groups (*P*-value is shown).

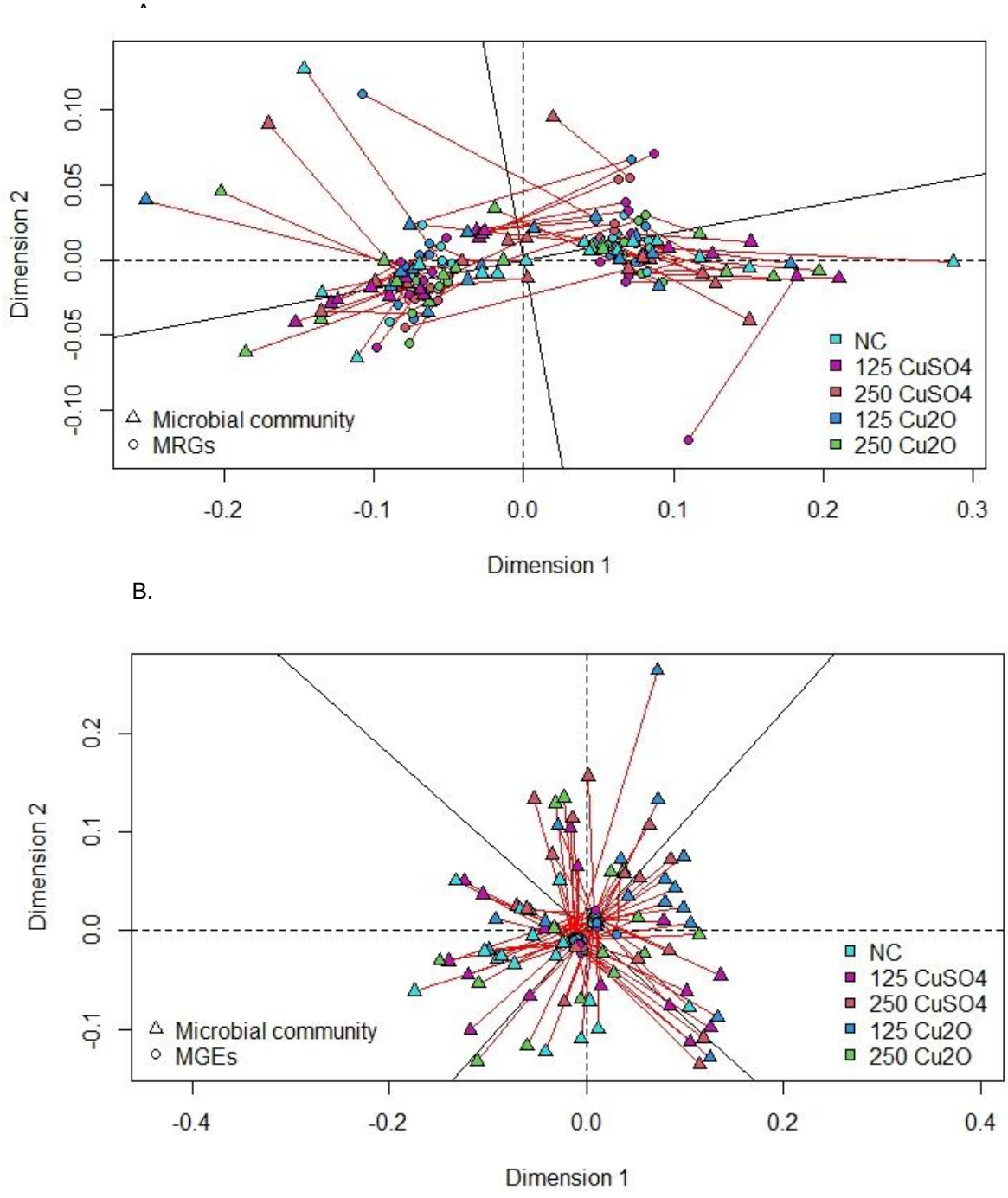


Fig. S12. Procrustes analysis showing the correlation between **A.** Metal resistance genes (MRGs) or **B.** Mobile genetic elements (MGEs) and bacterial community composition (16S rRNA gene amplicon sequence variants, ASVs) among all fecal samples analyzed by both HT-qPCR and amplicon sequencing (*n* = 80). The red lines connect the two data sets. Triangles refer to 16S rRNA gene ASV data and circles refer to MRG (panel A) or MGE (panel B) data. Samples are color-coded according to dietary Cu treatment: NC (negative control), 125 $CuSO_4$ (copper sulfate, 125 µg g^{-1}), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}), 125 Cu_2O (monovalent copper oxide, 125 µg g^{-1}) and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}).

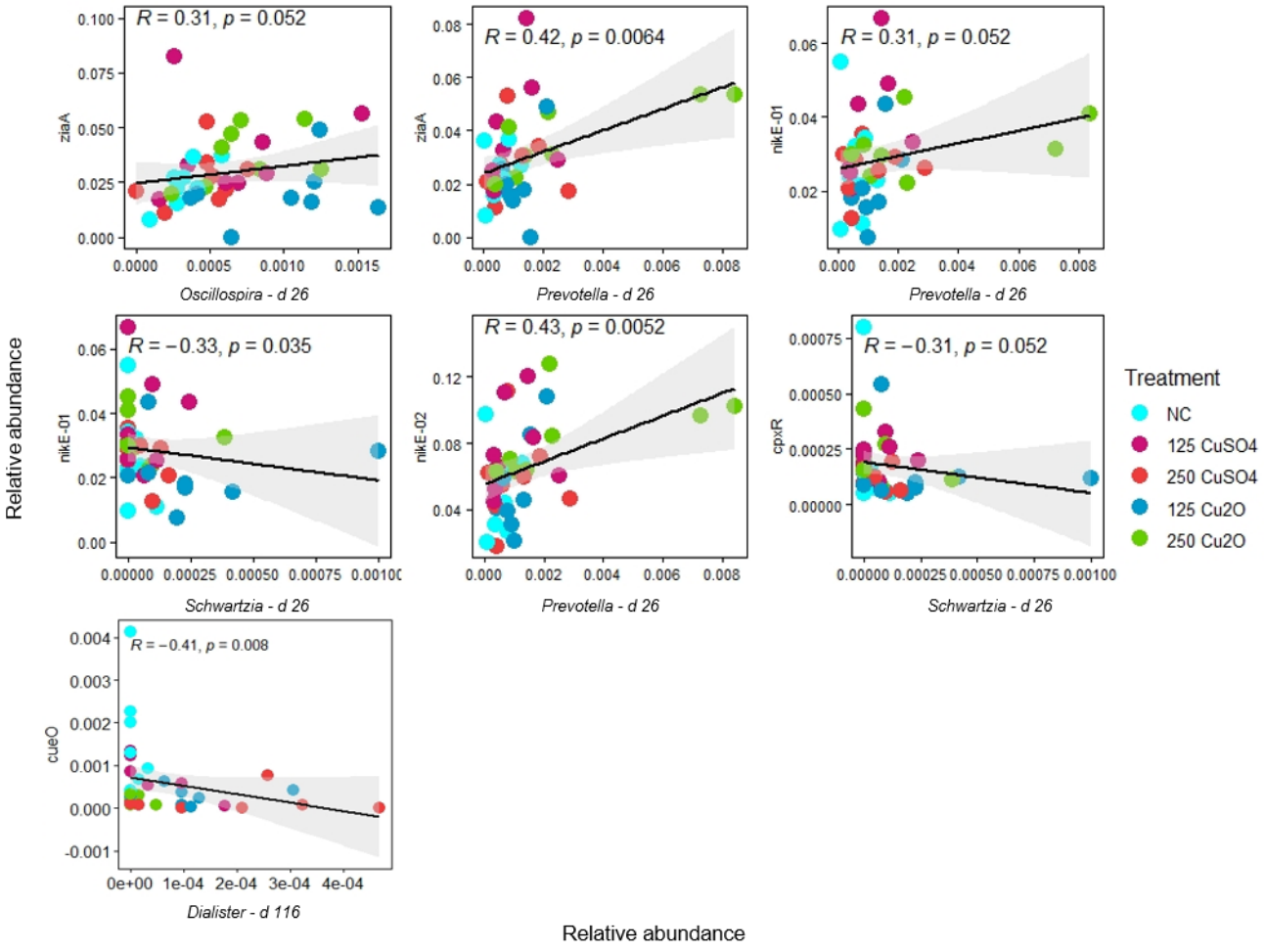


Fig. S13. Spearman's rank correlations between the relative abundance of MRGs or MGEs (genes/16S rRNA gene) and the relative abundance of bacterial genera on day 26 or 116 of the experiment.

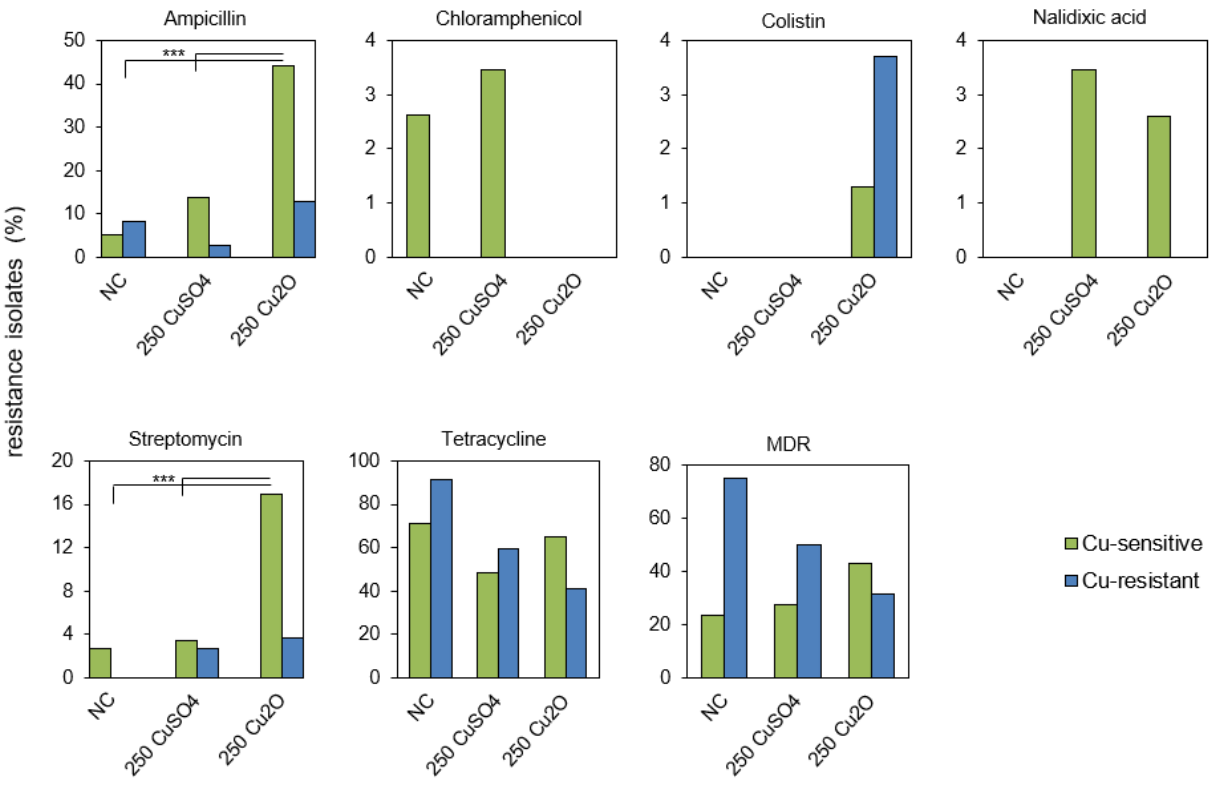


Fig. S14. Frequency of resistance to six antibiotics and multidrug resistance (MDR) among *E. coli* isolates obtained from different dietary Cu treatment groups: NC (negative control; 50 isolates), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}; 103 isolates), and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}; 131 isolates) based on Cu-sensitivity. Significant differences in antibiotics resistance between isolates from the NC group and both Cu treatments, as well as between the $CuSO_4$ and the Cu_2O groups were examined. The level of significance is indicated as follows: Generalized linear model (GLM); *** $P <$ 0.001.

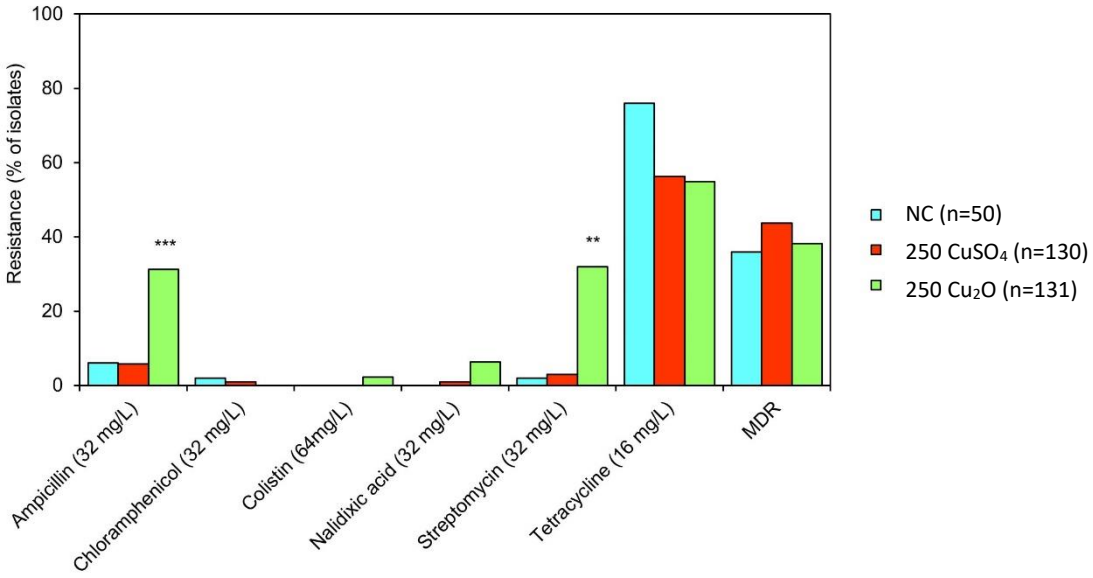


Fig. S15. Frequency of resistance to six antibiotics and multidrug resistance (MDR) among *E. coli* isolates (*n* = 284) obtained from different dietary Cu treatments: NC (negative control; 50 isolates), 250 $CuSO_4$ (copper sulfate, 250 µg g^{-1}; 103 isolates), and 250 Cu_2O (monovalent copper oxide, 250 µg g^{-1}; 131 isolates). Significant differences in resistance detected between isolates from the NC group and the Cu treatments were examined. The level of significance is indicated as follows: Generalized linear model (GLM); ***$P < 0.001$; **$P < 0.01$.

GENERAL DISCUSSION

1. Function: growth enhancement

In this thesis, we hypothesized that high concentrations of Cu result in improved growth performance as already proved by existing literature, however, an effective Cu source (i.e., Cu_2O) in broiler diets would have a greater impact on growth performance than the commonly used $CuSO_4$. We further assumed that Cu's antimicrobial action was responsible for this effect and that the disparity in Cu sources speciation would influence gut microbiota in distinct ways, resulting in varying impacts on growth performance. To evaluate the antimicrobial efficacy of Cu, we opted to conduct our experiment using previously used litter with elevated concentrations of *Clostridium perfringens*, which is known to cause necrotic enteritis.

The use of recycled litter in our experiment resulted in reduced growth performance compared to standard values. Typically, a 42-day old male ROSS 308 weighs around 3.02 kg, whereas our chickens had an average BW of 2.48 kg. Although the animals' BW in the Cu-supplemented groups were not significantly different from the NC group there was a notable increase from 15 mg/kg of Cu_2O to its therapeutic level (150 mg/kg) at day 35 and 42, something which was not observed with $CuSO_4$ addition. Supplementation of Cu from Cu_2O, irrespective of dose, tended to have lower ADFI than $CuSO_4$ supplementation and lower feed conversion ratio than $CuSO_4$ and non-supplemented diet at d 42. These observations may suggest the effectiveness of high doses of Cu_2O in promoting performance. As mentioned in the literature review, Cu has been reported to have the ability to promote feed intake, which in turn results in

increased BW of animals. However, our study presented different findings that cannot be dismissed.

Previously, our research group conducted a study on broilers using the same Cu sources and obtained similar results where Cu supplementation with Cu_2O at a therapeutic dose (150 mg/kg) increased BW without any changes in other growth performance indicators compared with 15 mg/kg dose (Hamdi et al., 2018). In this study, they also observed a decrease in phytic phosphorus solubility and a potentially toxic or corrosive response when $CuSO_4$ was supplemented.

An additional experiment performed in our group (Blavi et al., 2021), investigated the impact of these Cu sources on swine growth performance. The results of this study further supported the use of Cu_2O as a supplement. The findings showed that diets supplemented with Cu_2O had a positive effect on the growth performance and bone mineralization of growing pigs, while also leading to less Cu accumulation in the liver compared to diets containing $CuSO_4$.

The results of our experiment together with Hamdi et al. (2018) suggested that there may be reasons beyond increased ADFI (discussed in the literature review) for the observed performance enhancement in broilers supplemented with therapeutic levels of Cu_2O. In light of that, we proposed a potential mechanism through which Cu could promote growth, involving the modulation of intestinal microflora, as has been previously suggested by other researchers.

Our findings revealed that the addition of Cu to broiler diets, whether in the form of $CuSO_4$ or Cu_2O, led to changes in the composition of the gut microbiota. The supplementation of Cu_2O was more effective than $CuSO_4$ in decreasing the abundance of *Streptococcaceae* which includes several species acknowledged as opportunistic

pathogens (Munita et al., 2012; Qiao et al., 2014), and in increasing the presence of *Peptostreptococcaceae* (higher proportion found in the gut microbiota of healthy pigs; Fan et al., 2017) and *Clostridiaceae* which is found to be involved in butyrate production (Esquivel-Elizondo et al., 2017). Additionally, Cu_2O supplementation increased microbial diversity and evenness in the ileum. By reducing the abundance of families including pathogens, high Cu_2O supplementation may create a more favorable environment for the growth of other beneficial families. This may be attributed to the potency of Cu^+ (existed in Cu_2O) over Cu^{2+} (existed in $CuSO_4$) ion in inhibiting microorganisms (Saphier et al., 2018). These results may explain why Cu supplementation, particularly with Cu_2O, improves better performance traits in poultry, as it helps maintain a healthy gut balance.

Our study in pigs also revealed a similar trend in colon microbiota profile. Adding 250 mg/kg of $CuSO_4$ or Cu_2O to the diets increases the abundance of *Peptostreptococcaceae* and *Lachnospiraceae* families, and decreased the abundance of the *Rikenellaceae* family, *Campylobacter*, and *Streptococcus* genera. The impact of Cu supplementation from both sources versus the NC was more pronounced than the effect of Cu_2O compared to $CuSO_4$ on gut microbiota of swine. This finding alone is not sufficient to provide a clear explanation for the observed difference in growth performance with Cu supplementation. Other functions of Cu, such as its mechanistic role may have contributed to the differences in growth performance. Therefore, we conducted further investigations to explore any potential changes in other parts of the body.

2. Modes of action: systemic mechanisms

Our broiler experiment showed results similar to many animal studies that suggest Cu's growth-promoting effects are due to its antimicrobial properties in the GIT. However, we believe that enhanced growth performance is a result of a series of complex events rather than a simple microbial modulation. We also acknowledge that the source of Cu can affect its functionality in these events. Therefore, we conducted an experiment using samples collected from a previous study in our research group with pigs (Blavi et al., 2021). Our objective was to assess changes in parameters related to the oxidative status, inflammation, and gene expression as potential mechanisms of high Cu supplementation alongside gut microbiome modulation.

Genes are fundamental in determining an animal's traits, growth, development, and overall health. They contain vital genetic information that governs physical attributes and metabolic processes. Gene expression can be impacted by numerous factors, including nutrient intake and diet. Our study revealed that high Cu supplementation can modulate the abundance of genes accountable for improving intestinal epithelial barrier function, controlling immunological and inflammatory responses, and increasing nutrient absorption and feed intake. These findings indicate that Cu supplementation can have a considerable impact on gene abundance and can promote better animal health and growth.

Concurrently, an expanding body of research suggests that the microbiota plays a crucial role in regulating brain function and behavior through the microbiota-gut-brain axis, which enables gut microbes and the brain to communicate bidirectionally (Canfora et al., 2015; Foster et al., 2016; Rhee et al., 2009). In our experiment, we also observed

such a correlation between these factors, as pigs fed diets containing therapeutic levels of Cu exhibited reduced concentrations of pathogenic microbes followed by a lower abundance of immune response genes. Yet, additional research is required to fully understand the impact of Cu on gene expression within the microbiota-gut-brain axis and how this may relate to growth performance and overall health outcomes.

The high concentration of Cu can cause oxidative stress (previously discussed in the general overview), which may negatively affect pig health and growth performance. Our experiment showed that administering high levels of $CuSO_4$ (250 mg/kg) compared to Cu_2O resulted in increased liver oxidation levels, as indicated by MDA levels as a biomarker, and elevated pro-inflammatory cytokine levels in the bloodstream. These results suggest that $CuSO_4$ may cause tissue damage and trigger an inflammatory response, potentially leading to adverse effects on pig health. Therefore, Cu_2O may be a better Cu source for promoting growth performance without inducing excessive oxidative stress and inflammation.

While $CuSO_4$ readily dissolves in water and can react with other compounds in the digestive tract to form reactive oxygen species, Cu_2O's limited solubility could reduce the risk of oxidative stress and inflammation associated with its supplementation. Therefore, Cu_2O's lower prooxidant activity compared to $CuSO_4$ could be attributed to its limited solubility in water which could lead to improved growth performance without negative health consequences.

Although our results initially supported our hypothesis, this conclusion was based on data collected on day 26 of the experiment, where supplementation of 250 mg/kg of Cu from both sources led to higher BW compared to the non-supplemented diet (Blavi

et al., 2021). However, the performance results at the end of the experiment favored Cu_2O, which demonstrated better performance compared to all other treatments. Unfortunately, we did not have samples from the final day of the experiment (day 116) to further investigate and provide a clearer answer. Nonetheless, we suspect that differences in oxidation and inflammatory response between Cu sources may have contributed to this final observation.

3. Environmental impact: prevalence of antibiotic resistance

While various studies have highlighted the positive effects of Cu on animal performance, the negative impact of Cu on the environment has been overlooked in animal production studies. In our research, we sought to investigate the potential impact of high levels of Cu on the prevalence of antibiotic resistance in gut bacteria. We hypothesized an increase in the prevalence of specific genes linked to Cu resistance would be the reason behind gut bacterial antibiotic resistance, we also aimed to determine whether the differences in sources of Cu could mitigate its potential adverse effects on antibiotic resistance.

High dietary intake of Cu, like other metals, may have unintended consequences for the growth of Cu-resistant bacteria. Our experiment with broilers did not find a link between high Cu supplementation and the prevalence of Cu resistance gene, *tcrB,* or ARGs in *Enterococcus spp.* and *E. coli*, but phenotypic results were inconsistent. *Enterococcus spp.* from the non-supplemented group showed higher antibiotic resistance, while *E. coli* from the supplemented groups showed higher antibiotic resistance as expected. In fact, phenotypical resistance in these isolates was high with

almost all the antimicrobial agents, irrespective of dietary treatment. This study did not find any significant effect of high Cu supplementation on Cu resistance gene (CRG) or ARGs, indicating that using samples from a study with reused litter may not be an appropriate method for detecting ARGs.

Our pig experiment was therefore a good opportunity to look further into this issue without the role of reused litter. Surprisingly, no evidence for the co-selection of ARGs was found throughout the experiment (Brinck et al., 2023).

It was unclear from that study whether the high Cu diets were indeed high enough to directly induce Cu resistance in gut bacteria, or if co-selection of Cu and antibiotic resistance may have occurred in specific bacterial species within the microbiome. Our third study was designed to address this point using the same samples. Our results showed that high Cu supplementation had minimal impact on the swine gut bacterial metal resistome and it was primarily affected by gut microbiome maturation. While the phenotypic Cu resistance data indicated that high Cu diets could select for Cu-resistant bacterial strains, surprisingly, there was no evidence of co-selection of Cu resistance and antibiotic resistance in *E. coli*. Additionally, we did not find any significant difference between the two sources of Cu.

Our findings were unexpected as they do not support the notion that dietary Cu plays a role in shaping CRG profiles. The limited effects of Cu on the gut microbiome may be due to the long-term use of high levels of dietary Cu in pig breeding, resulting in stable integration of CRGs in the fecal microbiome. Instead, we observed a strong correlation between the profiles of CRGs and bacterial community composition. This is consistent with our previous study which demonstrated a similar correlation between ARG profiles and bacterial community composition (Brinck et al., 2023).

We suggest that the absence of evidence for co-selection of antibiotic resistance by Cu in our pig feeding trial (Brinck et al., 2023; third manuscript) can be attributed to the minimal effects of the dietary Cu treatments on the gut bacterial metal resistome observed in this study.

CONCLUSIONS

1. Supplementation of 150 mg/kg of Cu_2O compared with the low concentration (15 mg/kg) for 42 days in the diet was able to enhance the growth performance of broiler chickens raised under challenging conditions by necrotic enteritis. ADG increased in animals fed 150 mg/kg of Cu_2O compared with the non-supplemented diet without any significant difference in ADFI which to some extent can be explained by its microbial modulation effect suggesting its superiority as a performance-enhancing Cu source.

2. The enhancement in pig growth performance observed upon high-Cu (250 mg/kg) supplementation to diets is not solely attributed to the modulation of intestinal microbiota, but also due to the regulation of genes responsible for intestinal epithelial barrier function, immunological and inflammatory responses, feed intake, and nutrient absorption.

3. Supplementation of diets for growing pigs with 250 mg/kg Cu from $CuSO_4$ but not Cu_2O, can increase liver oxidation and biomarkers of inflammation in serum. This difference may be attributed to the high-water solubility of $CuSO_4$ as compared to Cu_2O.

4. The use of recycled litter, which may have already reached saturation levels of resistance, in the experiment setup may not provide an accurate representation of the impact of high-Cu addition on bacterial resistance.

5. The results of our pig feeding trial contradicted the hypothesis of Cu-induced co-selection of antibiotic resistance, which can be attributed, to the minimal effects of the administered dietary Cu treatments on the gut bacterial metal resistome. This

observation in general exempts the notion that different Cu speciation may have varying effects on the emergence of antimicrobial resistance.

6. Interestingly, the correlation between MRG profiles and bacterial community composition is more significant than the role of dietary Cu in shaping these profiles. Therefore, it is likely that other factors, such as bacterial community composition, play a more crucial role in shaping MRG profiles.

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Scientific outcomes of this Ph.D. thesis

Publications

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