Journal of Animal Science, 2022, **100**, 1–12
https://doi.org/10.1093/jas/skac224
Advance access publication 20 June 2022
Non Ruminant Nutrition

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

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Abstract

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 day 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 regulates genes abundance in charge of the immune system and growth, and promotes changes in the intestinal microbiota; however, Cu_2O induces less systemic oxidation and inflammation compared with $CuSO_4$.

Lay Summary

Copper is a nonrenewable mineral resource that is essential for all biological organisms. After banning the antibiotics, copper has received considerable attention due to its antimicrobial properties that improve performance in animals when fed over the minimum requirement. The present study evaluated two sources of Cu (copper sulfate and monovalent copper oxide) compared with a nonsupplemented diet and the likely mechanism of action which leads to improved pig performance. Pigs fed high concentrations of copper sulfate showed increased liver oxidation and inflammatory indicators in the blood. Elevated concentrations of Cu improved intestinal epithelial barrier function, modulation of inflammatory responses, increased beneficial microbes, and reduced pathogens in the gut. Therefore, supplementation of high levels of Cu appears to be effective in promoting pig growth, but therapeutic doses of Cu sulfate increase the inflammatory response.

Key words: copper sulfate, gene abundance, monovalent copper oxide, microbiota, oxidative status, swine

Abbreviations: BF, barrier function; EH, enzymes and hormones; GSH-Px, glutathione peroxidase; IR, immune response; MDA, malondialdehyde; NT, nutrient transport; RNA, ribonucleic acid; ST, stress; SOD, Cu/Zn superoxide dismutase

Introduction

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 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 al., 1989; Hill et al., 2000), and the

Received February 25, 2022 Accepted June 17, 2022.
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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, 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.

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 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).

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.

Diets and feeding

Three diets based on corn and soybean meal were formulated (Table 1). Diet contained 500 units of phytase per kilogram (Quantum Blue, AB Vista Feed Ingredients,

Table 1. 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	20.00
Analyzed composition, %	
Dry matter	87.19
Ash	4.07
Crude protein	17.88
Lys	1.38
Ether extract	5.50

[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.

Malborough, UK). Dietary treatments consisted of the negative control diet (NC) with 20 mg/kg of added Cu, and two 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).

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 two ethylenediaminetetraacetic acid (EDTA) vacutainers (BD Diagnostics, Franklin Lakes, NJ). To recover serum, vacutainers were centrifuged at 700 × *g* at 4 °C for 13 min. One set of serum samples was stored at −20 °C until analyzed for cytokines and chemokines. The other set of serum samples was stored at −80 °C until analyzed for malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and Cu/Zn superoxide dismutase (SOD) activity.

Pigs from which blood samples were collected were killed to obtain liver, jejunum, and colon digesta. Liver samples were collected and immediately placed in liquid nitrogen and stored at −80 °C 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 −80 °C 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.

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 (0.32 M) 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 4 °C 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.

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), and 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).

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) as explained by González-Solé et al. (2020).

Microbiota 16S rRNA gene analysis

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′-TCGTCGGCAGCGTCAGATGTGTATAAGAG-ACAGCCTACGGGNGGCWGCAG-3′, V3-V4-Reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). The library preparation was performed in Microomics Systems SL (Barcelona, Spain).

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).

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 $0.05 \le P < 0.10$.

Gene abundance data were analyzed using the ThermoFisher Cloud software 1.0 (Applied Biosystems, Waltham, MA) 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 functions. 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.

Results

Oxidative stress markers and malondialdehyde concentrations

Malondialdehyde, SOD, and GSH-Px in the serum were not different among dietary treatments (Table 2). 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 2. Mean plus/minus standard deviation values of 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]

Item	NC[2]	$CuSO_4$	Cu_2O
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 shown as the mean ± SD with 8 observations per treatment.
[2]NC: negative control; $CuSO_4$: 250 mg Cu/kg from $CuSO_4$; Cu_2O: 250 mg Cu/kg from Cu_2O.
[a,b]Means with different subscripts within a row differ ($P < 0.05$).
[x,y]Means with different subscripts within a row differ ($P < 0.10$).

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 1). 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.

Gene abundance

A heatmap was constructed to observe overall similarities among gene abundance profiles (Figure 2). 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 3). 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.

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,

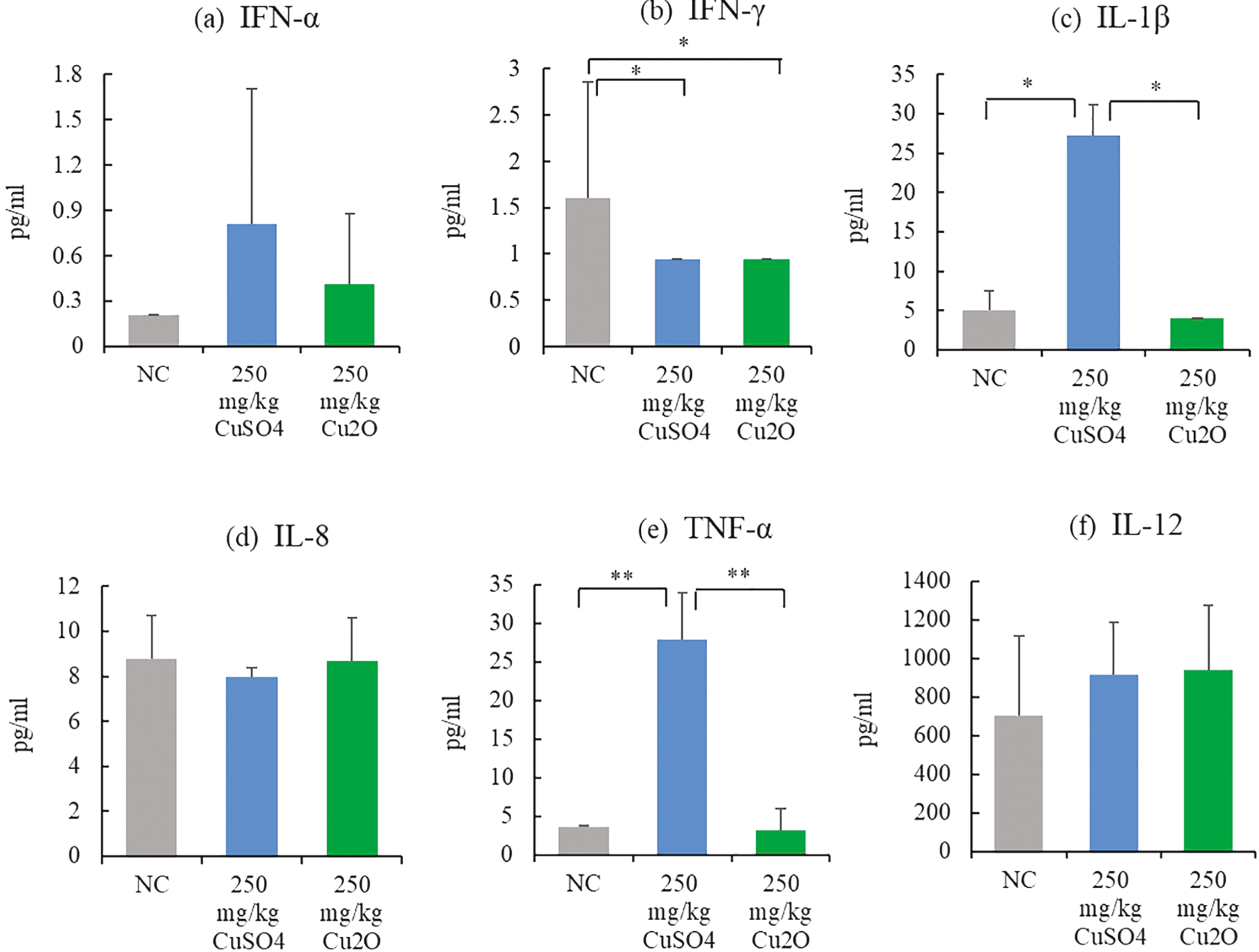

Figure 1. Cytokine concentration in the serum of pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O. Data are means of 8 observations per treatment. Error bars show the standard deviation. PROC MIXED significance, * indicates $P < 0.1$ and ** indicates $P < 0.05$.

Euryarchaeota, Fibrobacteres, Firmicutes, Kiritimatiellaeota, Lentisphaerae, Patescibacteria, Planctomycetes, Proteobacteria, Spirochaetes, Synergistetes, and Tenericutes were identified (Figure 3a). 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 3b.

The following families were different (Figure 4) 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.

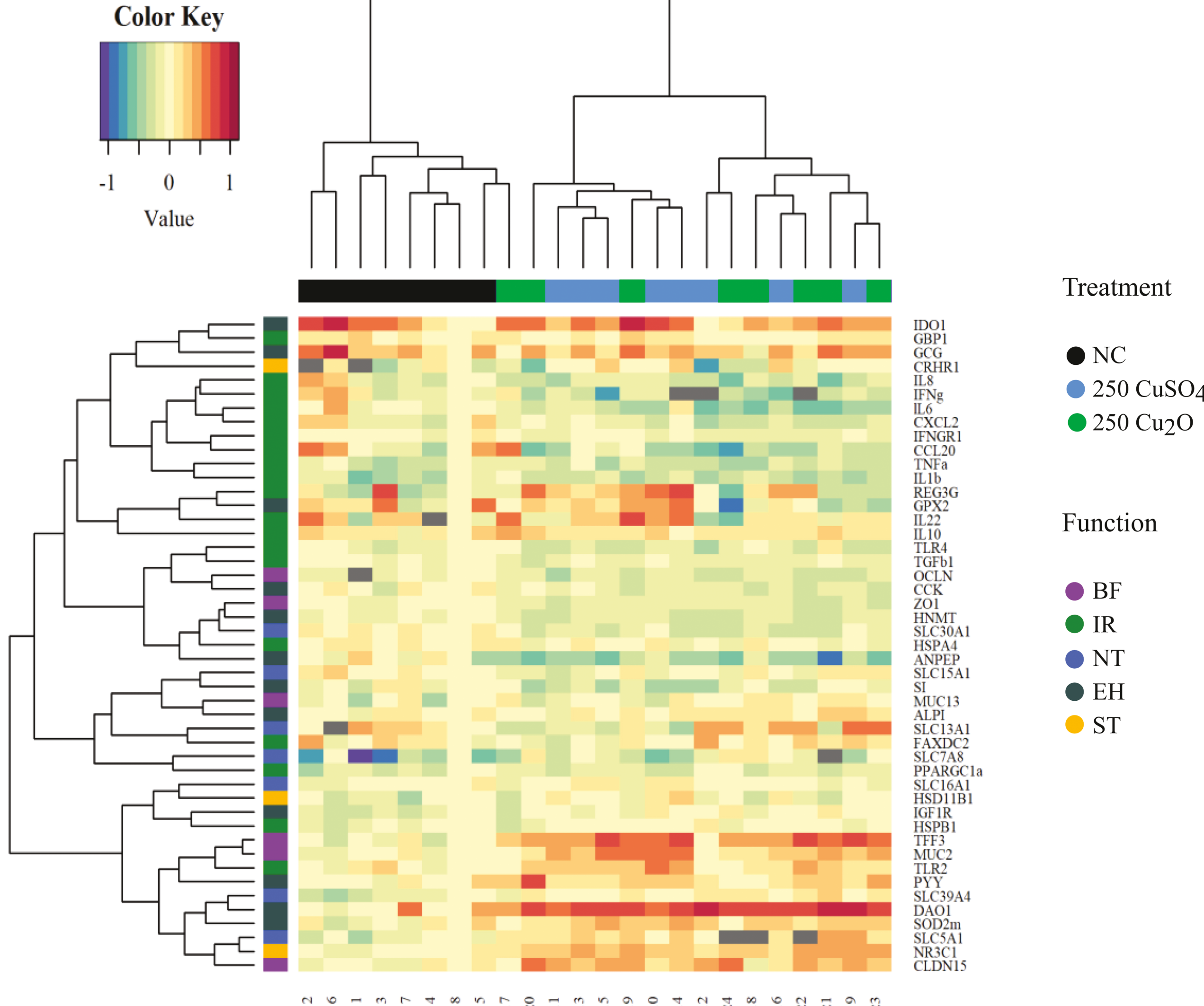

Figure 2. Heatmap representing the most abundant genes (n = 47) related to barrier function (BR), immune response (IR), nutrient transport (NT), enzyme/hormone (EH), and stress (ST). The color gradient represents the relative gene abundance. Rows represent the results of each gene shown on the y-axis. Columns represent tissue samples (n = 24) related to each treatment: negative control (NC), 250 mg Cu/kg from $CuSO_4$, and 250 mg Cu/kg Cu_2O.

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.

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

Table 3. 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[3]	$CuSO_4$	Cu_2O	Contrast Statistic[4]	*P*-value	*Q*-value (FDR)
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
	lL-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]NC: negative control; $CuSO_4$: 250 mg Cu/kg from $CuSO_4$; Cu_2O: 250 mg Cu/kg from Cu_2O.
[4]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 ($P < 0.05$)

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* was 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

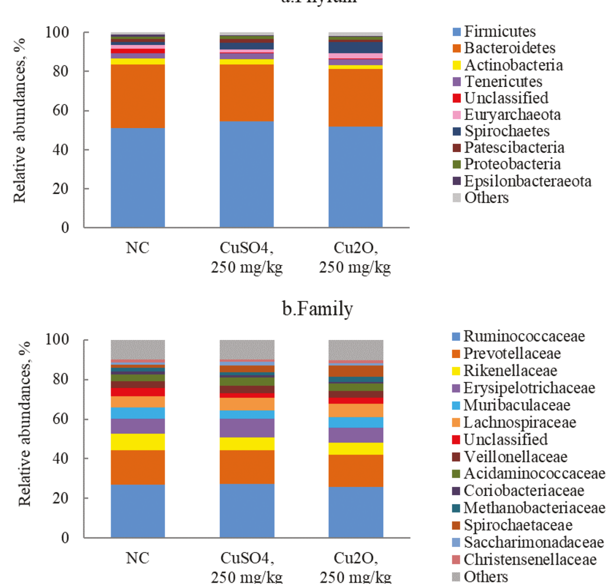

Figure 3. Relative abundance (%) of phyla (a), and families (b) present in the colon microbiota of pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O. The rest of the taxonomic groups are pooled together (those representing less than a mean of 1% of phyla and families).

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.

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 (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).

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, 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

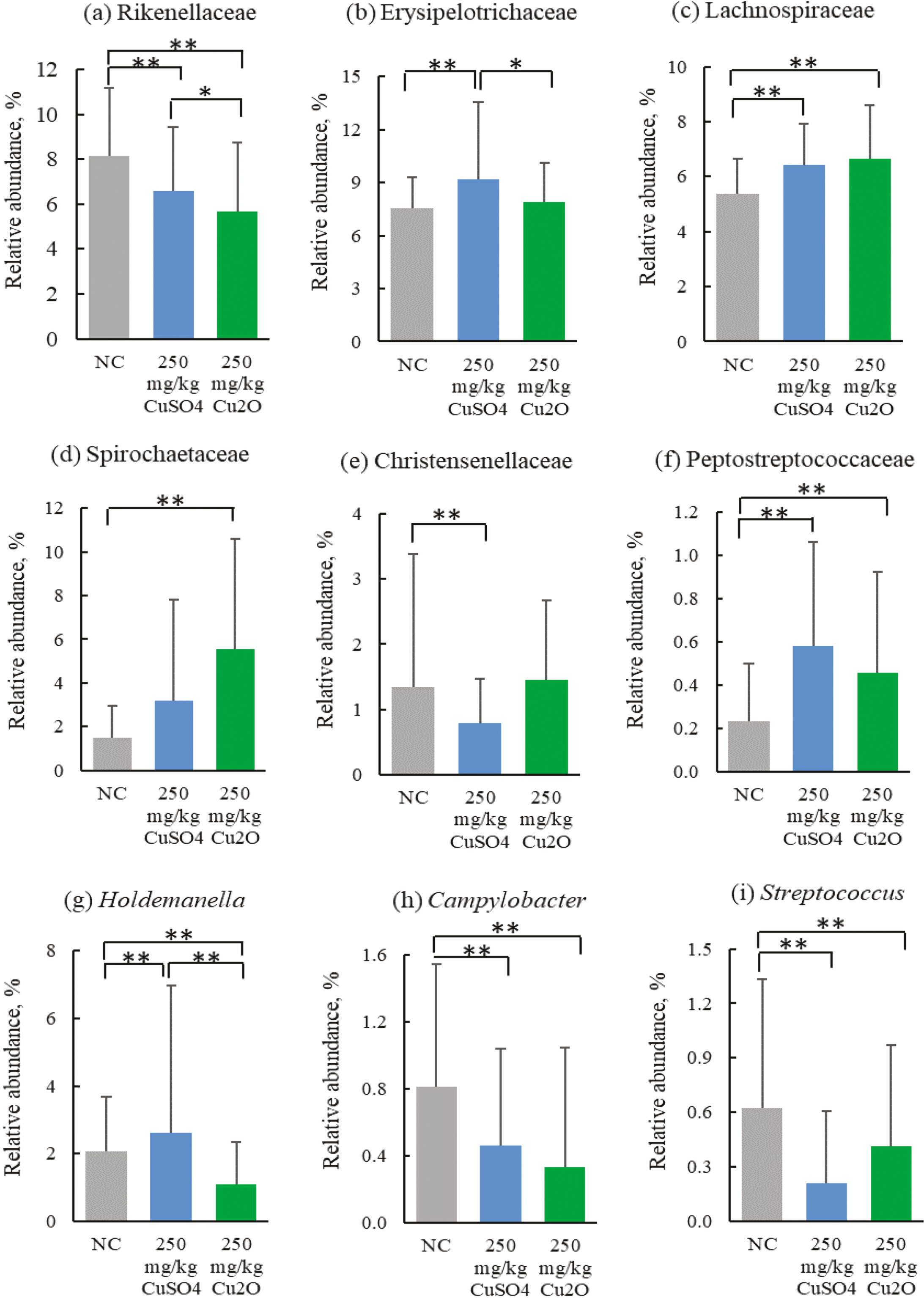

Figure 4. Colonic bacterial families and genera whose abundance significantly differs between pigs fed control diet and diets with 250 mg Cu/kg from $CuSO_4$ or Cu_2O. Data are means of 8 observations per treatment. Error bars show the standard deviation. ANOVA significance, *indicates $P < 0.1$, and ** $P < 0.05$.

(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.

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.

Acknowledgments

The financial support from Animine, Annecy, France, is greatly appreciated. This work was also supported by Agéncia de Gestió d'Ajusts Universitaris i de Recerca de la Generalitat de Catalunya (PhD grant 2019FI_B 00282). David Solà-Oriol is supported by the UAB-Banco de Santander Talent Program.

Conflict of Interest Statement

Alessandra Monteiro is an employee at Animine, Annecy, France, a company that has commercial interests in the mineral nutrition of food-producing animals. Animine adheres to the principles of the European Code of Conduct for Research Integrity (Drenth, 2010). Laia Blavi is an employee of AB Neo. All other authors have no conflict of interest.

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