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Original Research Article

Different copper sources and levels affect growth performance, copper content, carcass characteristics, intestinal microorganism and metabolism of finishing pigs

Yang Wen [a], Runxian Li [a], Xiangshu Piao [a], Gang Lin [b], Pingli He [a, *]

[a] State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China
[b] Institute of Quality Standards and Testing Technology for Agricultural Products, Chinese Academy of Agricultural Science, Key Laboratory of Agrifood Safety and Quality, Ministry of Agriculture, Beijing 100081, China

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ABSTRACT

Copper (Cu) is an essential trace element in the production of swine. This study was conducted to investigate the effect of 3 different sources of Cu on growth performance, Cu metabolism, and intestinal microorganisms of finishing pigs, so as to estimate the bioavailability of the 3 sources for pigs. A total of 42 male finishing pigs (88.74 ± 5.74 kg) were randomly allocated to 7 treatments. The factors were 3 sources ($CuSO_4$, Cu-glycine, Cu-proteinate) and 2 levels (5 and 20 mg/kg) of Cu, plus one negative control treatment (0 mg/kg added Cu level) for the entire 28-d experiment. The average daily gain (ADG) and feed to gain ratio (F:G) both increased when Cu was added. The Cu level in liver, bile, kidney, serum, lung, urine and feces rose ($P < 0.001$) with increasing dietary Cu level regardless of the source. Meanwhile, pigs receiving organic Cu (glycinate or proteinate) retained more Cu and excreted less Cu than those receiving inorganic Cu ($CuSO_4$), which showed that organic forms were more bioavailable. At the transcriptional level, changes in the level and source of dietary Cu resulted in modulation of transporters. In the jejunal mucosa, import transporter high affinity copper uptake protein 1 (*CTR1*) and export transporter ATPase copper transporting alpha (*ATP7A*) in supplemental Cu treatments were down-regulated compared to the control. Also, peptide transporter 1 (*PepT1*) and lanine-serine-cysteine transporter, type-2 (*ASCT2*) were significantly ($P < 0.01$) up-regulated in 20 mg/kg Cu-proteinate and Cu-glycinate treatments, respectively. Microbial diversity was lowest in the 20 mg/kg $CuSO_4$ treatment, and the ratio of Firmicutes to Bacteroidetes was higher in added Cu treatments, especially Cu-glycinate treatment. These results indicate that uptake of different Cu forms is facilitated by different transporters and transport mechanisms, and compared with inorganic Cu, organic Cu provides benefits to intestinal microflora and reduces Cu excretion.

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1. Introduction

Copper (Cu) is an essential trace element, which plays an important part in maintaining healthy and productive swine (Gaetke et al., 2014). It also prevents oxidative stress and diarrhea and promotes the growth of pigs and supports immune system function (Ian and Beattie, 1995). Because of these crucial functions, Cu is typically supplemented to piglets at high levels (125 to 250 mg/kg) (Coble et al., 2017; Shelton et al., 2011; Zhao et al., 2008; Guo et al., 2001). However, negative effects of high Cu are becoming more apparent. For example, chronic supplementation of high Cu may lead to a risk of Cu accumulation in pigs leading to damage of

* Corresponding author.
E-mail address: hepingli@cau.edu.cn (P. He).
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the liver, kidneys, and intestinal mucosa (Sanchez et al., 2005). Simultaneously, high residual Cu accumulation in edible animal tissues can be a hazard to human health, like Alzheimer's disease (Brewer, 2015). High Cu levels in excrement can also cause serious environmental risks (Zhang et al., 2011; Sanchez et al., 2005; Miles RD, 1998). To address these potential problems, China has regulated that dietary Cu for finishing pigs (>25 kg) should not exceed 25 mg/kg in feed (China, 2018). It is worth noting that the NRC (2012) set the dietary Cu requirement at 5 to 10 mg/kg for pigs.

In the swine industry, the most commonly used Cu supplements are Cu sulfate ($CuSO_4$) and Cu oxide which are inorganic sources. However, because of the poor Cu bioavailability of these sources, many alternatives have been found. Many studies have demonstrated that organic Cu from copper glycine (Cu-Gly) and copper proteinate (Cu-Pro) have greater bioavailability compared to inorganic Cu (Huang et al., 2010; Veum et al., 2004). For example, Cu-Pro is a number of Cu complexes with oligopeptides (3 to 10 amino acids) by the chelation of a soluble Cu salt with enzymatically hydrolyzed soy protein, which appeared to be more effective in enhancing the growth rate of weanling piglets, and reducing Cu excretion (Lin et al., 2020). At present, organic Cu is popular for use in swine diets because there are fewer negative consequences compared with feeding inorganic trace minerals (Ma et al., 2018).

Absorption of organic Cu in pigs is different from inorganic Cu, though the exact metabolic pathways for absorption are still debated. In general, the Cu compound enters the digestive tract and is hydrolyzed on the brush border of the intestine. Cu ions are then absorbed into the blood through intestinal epithelial cells. After processing, Cu is bound to albumin and transferred to other tissues. Excess Cu is excreted mainly through the biliary tract (Miyayama et al., 2010). Cu homeostasis is maintained by many chaperone proteins and transport carriers (Kim et al., 2008). For example, high affinity copper uptake protein 1 (CTR1) is a high-affinity Cu transporter which is located on cell membranes to uptake extracellular Cu ions (Lee et al., 2002). Antioxidant 1 copper chaperone (*ATOX1*) encodes a Cu chaperone that plays a role in Cu homeostasis by binding and transporting cytosolic Cu to ATPase proteins in the trans-Golgi network for later incorporation to ceruloplasmin (Pereira et al., 2016; Fontaine and Mercer, 2007). Additionally, peptide transporter 1 (PepT1) and the lanine-serine-cysteine transporter, type-2 (ASCT2) are closely related to the transport of Cu ligands, which may affect the transport of Cu.

The effect of intestinal microbiota on nutrient uptake and maintaining healthy growth is broadly known (Keenan et al., 2018). The gut microbiota changes that occur are related to animal production phase and feed composition. Some studies indicate that high Cu diets can change the gut microenvironment and affect the composition and abundance of bacteria (Di Giancamillo et al., 2018; Zhang et al., 2017). Moreover, antibacterial properties of high levels of dietary Cu can alter intestinal microbial diversity and composition in pigs (Di Giancamillo et al., 2018; Shurson et al., 1990). The effect of organic Cu on the intestinal microbiome is less studied, but the dissociation of Cu from the organic carrier molecule(s) is likely to result in alterations.

It is vital to maximize the biological efficacy of Cu and ensure the safety of animal products under the new Cu limit regulation for pigs in China. To our knowledge, the differences in specific metabolic processes and related mechanisms among organic and inorganic Cu sources in pigs have not been reported. The objective of this experiment was to study the effect of 3 different Cu sources ($CuSO_4$, Cu-glycine, Cu-proteinate) on growth performance, carcass yield, meat quality, Cu residues, microflora community and the expression of various genes in finishing pigs, and further explore the metabolic pathways that cause their differential function.

2. Materials and methods

All animals used in this study were managed according to the Chinese Guidelines for Animal Welfare. The experimental protocol was approved by the Animal Care and Use Committee of the China Agricultural University (Beijing, China).

2.1. Animal, diets and experimental design

Forty-two barrows (Duroc × [Landrace × Large White]) with an average body weight of 88.74 ± 5.74 kg were distributed randomly into 7 treatment treatments ($n = 6$) for a 28-d experimental period. The experiment used a 3 × 2 + 1 treatment structure. A corn-soybean meal diet (Appendix Table 1) and a vitamin mineral premix were prepared with no added Cu. Three supplemental Cu sources: $CuSO_4$ (cupic sulfate anhydrous, analytical reagent 99%, Aladdin, Shanghai, China), Cu-Gly (Cupic glycine chelate, 24% Cu, Pancosma, Shanghai, China) and Cu-Pro (10% Cu, Bioplex Cu, Alltech Inc., Nicholasville, KY, USA) were used. The control treatment (0 mg/kg Cu) was fed this basal diet. Test dietary treatments were supplemented with $CuSO_4$, Cu-Gly, or Cu-Pro at 5 or 20 mg added Cu/kg feed, resulting in 7 diets. The target supplementary low dietary Cu level was 5 and 20 mg/kg for high Cu (Table 1). Water was freely available from a low-pressure drinking nipple. All pigs were given ad libitum access to feed and water.

2.2. Housing and metabolism measurements

Pigs were housed individually in metabolism cages (1.4 m × 0.7 m × 0.6 m). Five days before harvest, complete collection of feces and urine was used to analyze Cu excretion. At 28 d, all 42 pigs were sacrificed, and the following samples were collected: tissue samples (liver, kidney, heart, spleen, lung, longissimus dorsi); serum and bile were collected to determine Cu concentrations; liver and jejunum mucosa were used to determine the expression of transporters; digesta from the proximal colon was used to analyse microbiota and short chain fatty acids.

2.3. Growth performance

Pigs were weighed individually on d 0, 24 and 28. Surplus feed was recorded per cage, and the total feed intake (FI), average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F:G) were calculated.

2.4. Carcass characteristics measurement

Hot carcass weight was recorded to calculate the dressing percent. The loin eye muscle area and backfat thickness were measured by a Vernier caliper (Mitutoyo, NTD13-P15M). The loin eye muscle area was determined at the 10th rib. The backfat thickness was determined as the mean of measurements taken at the 10th rib, shoulder and lumbar vertebra.

Table 1
Analyzed copper concentrations (mg/kg)[1] of complete diets [2].

Dietary copper source	Added copper level, mg/kg	
	5	20
Copper sulfate	12.16	26.39
Copper glycinate	15.04	27.08
Copper proteinate	11.25	25.89

[1] Copper element content.
[2] Intrinsic copper level of feedstuffs is 7.2 mg/kg.

2.5. Meat quality analysis

Meat quality was detected on the longissimus dorsi of the 10th rib. The pH was measured at 45 min and 24 h postmortem with a pH meter (Testo 205). The measurements of drip loss percentage and cooking loss were performed as the methods described by Xu et al. (2020).

2.6. Chemical analysis

The organs, tissues, blood, feces, urine and diets were analyzed for Cu content using inductively coupled plasma-mass spectroscopy (ICP-MS, Agilent) according to methods described by Li et al. (2020). The short-chain fatty acids in colonic digesta were determined by an ion chromatographic analyzer (ICS-3000, Dionex, U.S.) according to the methods described by Han et al. (2019).

2.7. Real-time quantitative PCR

Primers were designed with Primer 5.0 software using *Sus scrofa* sequences from the NCBI database and synthesized by Beijing Sunbiotech Co. Ltd. The primers used for quantifying selected genes are listed in Appendix Table 2. Firstly, 30 to 40 mg of sample were homogenized at 4 °C. The system for RNA extraction, reverse transcription and real-time quantitative PCR was performed according to Li et al. (2020). In addition, β-actin was used as an endogenous control. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blots

Total tissue protein lysates were extracted with radioimmunoprecipitation assay (RIPA) tissue lysis (Solarbio) that contained protease inhibitor and protein phosphatase inhibitor. Approximately 50 mg of total tissue lysates were loaded per tube. The relative protein expression of CTR1 and PepT1 was analyzed by Western blots.

Samples (50 μg of total protein) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer to polyvinylidene fluoride (PVDF) membrane (GE Health Care). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated overnight at 4 °C with the antibodies: rabbit anti-CTR1 (1:1,000, Genetex, GTX30642), rabbit anti-PepT1 (1:1,000, Bioss, bs-0689R) and rabbit anti-actin (1:1,000, Absin, abs132001). After washing 3 times with TBST, the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody (CST, 5151S) at 37 °C for 1 h. After washing 3 times with TBST, membranes were detected using clarity-enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific). Photoshop software was used to analyze the gray value of protein bands and target proteins.

2.9. Analysis for microbial community by 16S rRNA sequences

Twenty-four fresh colonic digesta samples of pigs in 4 dietary treatments (control, 20 mg/kg $CuSO_4$, 20 mg/kg Cu-Gly, 20 mg/kg Cu-Pro) were used to evaluate the microflora community ($n = 6$). Total genomic DNA was extracted from colonic digesta using a QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Final DNA concentration and purification were determined by NanoDrop (2000) UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3 to V4 region of the bacterial 16S ribosomal RNA gene was amplified by PCR that used the bacterial universal primers of 338F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with an eight-base sequence unique to each sample as a barcode. The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST (Promega, USA) according to the manufacturer's protocols. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego,USA) according to standard protocols (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China). Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH. Operational taxonomic units (OTU) were defined as a similarity threshold of 0.97 using UPARSE. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16SrRNA database using a confidence threshold of 70%. According to the guidance of R software, we used standardized OTU reads to analyze bacterial diversity by principal co-ordinates analysis (PCoA). We used the method of Kruskal–Wallis to analyze populations of the bacterial community in fecal samples of pigs at the phylum and family levels.

2.10. Statistical analysis

Data organization, scientific graphing, and statistical analyses were performed using Microsoft Excel (Redmond, WA, USA), GraphPad Prism (v.6; La Jolla, CA, USA), and SAS (version 9.2; SAS Inst. Inc., Cary, NC), respectively. Data for performance, Cu content, and meat quality were analyzed as a randomized complete block design using the MIXED model procedure of SAS version 9.2 (with the individual pig as the experimental unit). For responses in which the interaction was significant, only the interaction is discussed, whereas main effects means are discussed in cases where the interaction was not significant. Data for q-PCR and SCFA concentrations were subjected to ANOVA using the GLM procedure of SAS. Significantly different means (for interaction effects) were separated using Tukey. Significant differences were declared at $P < 0.05$.

3. Results

3.1. Growth performance and carcass characteristics

There was no significant source × level interaction or main effect of source on any of the growth performance parameters. Added Cu level significantly affected ADG ($P = 0.004$) and F:G ($P = 0.009$) (Table 2) such that ADG and F:G improved with added Cu, though there was not a significant difference between the 5 and 20 mg/kg Cu levels. Likewise, there were no significant main effects or interactions in carcass characteristics. However, the fat cover at the tenth rib ($P = 0.058$) tended to be higher when Cu was added at either 5 or 20 mg/kg as compared to the control. In addition, a significant main effect of level on marbling score and intramuscular fat increased when Cu was added (Appendix Table 3).

3.2. Cu distribution and deposition

There was no significant source × level interaction on Cu content except for a trend ($P = 0.078$) for fecal Cu (Table 3). The interaction shows that there was no significant difference for fecal Cu content among the control and all sources of Cu at 5 mg/kg treatments. When Cu was added at 20 mg/kg, fecal Cu excretion was elevated for all sources and reached the highest level with 20 mg/kg $CuSO_4$. Further, a significant main effect of Cu sources on the Cu contents in fecal, bile and liver samples was observed. Fecal Cu concentration in pigs receiving organic Cu (Cu-Pro, Cu-Gly) was

Table 2
Effects of different copper (Cu) sources and levels on growth performance and carcass characteristics of finishing pigs.

Cu source	Added Cu level, mg/kg	ADFI, kg	ADG, kg	F:G	Dressing percentage, %	Eye muscle area, cm^2	Shoulder, cm	Tenth rib, cm	Lumbar vertebra, cm	Average backfat, cm
$CuSO_4$	0	2.91	0.93	3.18	82.00	34.10	4.43	2.44	1.44	2.78
	5	3.02	1.04	2.92	82.00	37.60	4.31	2.71	1.91	2.98
	20	3.01	1.06	2.88	81.00	36.32	4.39	2.95	1.83	3.05
Cu-Gly	0	2.91	0.93	3.18	82.00	34.10	4.43	2.44	1.44	2.78
	5	2.91	1.03	2.82	80.00	32.52	4.34	2.86	1.68	2.96
	20	3.02	1.01	3.01	84.00	31.76	4.40	2.73	1.77	2.95
Cu-Pro	0	2.91	0.93	3.18	82.00	34.10	4.43	2.44	1.44	2.78
	5	3.12	1.08	2.92	82.00	33.48	4.67	2.85	1.62	3.04
	20	3.10	1.04	3.00	82.00	33.01	4.40	2.77	1.80	2.99
SEM		0.09	0.05	0.12	0.01	1.60	0.24	0.21	0.21	0.20
P-value for source × level		0.809	0.943	0.899	0.896	0.503	0.914	0.918	0.954	0.997
Main effect of source										
$CuSO_4$		2.98	1.01	2.99	81.67	36.01	4.38	2.70	1.73	2.94
Cu-Gly		2.95	0.99	3.00	82.00	32.79	4.39	2.68	1.63	2.90
Cu-Pro		3.04	1.02	3.03	82.00	33.53	4.50	2.68	1.62	2.94
SEM		0.05	0.03	0.07	0.01	1.22	0.14	0.12	0.12	0.11
P-value for source		0.399	0.780	0.914	0.889	0.087	0.805	0.991	0.782	0.958
Main effect of level										
	0	2.91	0.93^{b}	3.18^{a}	82.00	34.10	4.43	2.43	1.44	2.78
	5	3.02	1.05^{a}	2.89^{b}	81.33	34.53	4.44	2.81	1.74	2.99
	20	3.04	1.04^{a}	2.96^{ab}	82.33	33.70	4.40	2.80	1.80	3.00
SEM		0.05	0.03	0.07	0.01	0.92	0.14	0.12	0.12	0.11
P-value for level		0.152	0.004	0.009	0.251	0.814	0.986	0.058	0.118	0.997

ADFI = average daily feed intake; ADG = average daily gain; F:G = feed to gain ratio; $CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; SEM = standard error of the mean.
[a,b]Means in the same column with different superscripts differ ($P < 0.05$, $n = 6$).

Table 3
Effects of different copper (Cu) sources and levels on excrement, serum, bile and different tissues copper contents of finishing pigs.

Cu source	Added Cu level, mg/kg	Feces, mg	Urine, mg	Serum, mg/kg	Bile, mg/kg	Heart, mg/kg	Kidney, mg/kg	Spleen, mg/kg	Muscle, mg/kg	Liver, mg/kg	Lung, mg/kg
$CuSO_4$	0	163.32^{c}	1.72^{b}	1.89^{b}	5.30^{c}	14.61	16.32^{b}	4.33	1.59	101.00^{d}	4.21
	5	166.87^{c}	2.09^{ab}	2.16^{ab}	6.12^{c}	14.49	23.88^{ab}	4.79	1.79	101.76^{cd}	4.47
	20	427.37^{a}	3.8^{a}	2.67^{a}	6.87^{bc}	14.24	29.63^{a}	4.46	1.72	122.49^{bc}	5.76
Cu-Gly	0	163.32^{c}	1.72^{b}	1.89^{b}	5.30^{c}	14.61	16.32^{b}	4.33	1.59	101.00^{d}	4.21
	5	158.65^{c}	1.51^{b}	1.86^{b}	7.35^{abc}	14.33	25.32^{a}	4.48	1.8	124.41^{ab}	4.42
	20	348.94^{b}	2.68^{ab}	2.13^{ab}	9.21^{a}	15.73	26.45^{a}	4.84	1.97	137.88^{a}	5.13
Cu-Pro	0	163.32^{c}	1.72^{b}	1.89^{b}	5.30^{c}	14.61	16.32^{b}	4.33	1.59	101.00^{d}	4.21
	5	171.54^{c}	2.47^{ab}	2.13^{b}	7.06^{bc}	14.43	24.57^{a}	4.57	1.96	105.75^{bcd}	4.99
	20	333.21^{b}	2.93^{ab}	2.33^{ab}	8.53^{ab}	16.47	26.95^{a}	4.72	1.73	133.71^{a}	4.52
SEM		19.56	0.39	0.13	0.45	1.01	1.69	0.31	0.14	4.47	0.36
P-value for source × level		0.078	0.376	0.208	0.157	0.777	0.712	0.857	0.654	0.091	0.174
Main effect of source											
$CuSO_4$		252.52^{a}	2.53	2.24	6.01^{b}	14.45	23.28	4.53	1.70	108.42^{b}	4.81
Cu-Gly		223.64^{b}	1.97	1.96	7.29^{a}	14.89	22.70	4.55	1.79	121.10^{a}	4.59
Cu-Pro		222.69^{b}	2.37	2.12	6.88^{ab}	15.17	22.61	4.54	1.76	113.49^{ab}	4.57
SEM		11.29	0.22	0.07	0.26	0.58	0.98	0.18	0.08	2.58	0.21
P-value for source		0.047	0.195	0.059	0.008	0.675	0.873	0.995	0.741	0.005	0.659
Main effect of level											
	0	163.32^{b}	1.72^{b}	1.89^{b}	5.30^{c}	14.61	16.32^{b}	4.33	1.59	101.00^{c}	4.21^{b}
	5	165.69^{b}	2.02^{b}	2.05^{b}	6.84^{b}	14.42	24.59^{b}	4.61	1.85	110.64^{b}	4.63^{ab}
	20	369.84^{a}	3.14^{a}	2.38^{a}	8.20^{a}	15.48	27.68^{a}	4.67	1.81	131.36^{a}	5.14^{a}
SEM		11.29	0.22	0.07	0.26	0.58	0.98	0.18	0.08	2.58	0.21
P-value for level		<0.001	<0.001	<0.001	<0.001	0.395	<0.001	0.364	0.654	<0.001	0.011

$CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; SEM = standard error of the mean.
[a,b,c,d]Means in the same column with different superscripts differ ($P < 0.05$, $n = 6$).

lower than those receiving $CuSO_4$. The contents of Cu in liver and bile were in the order of Cu-Gly > Cu-Pro > $CuSO_4$ treatment. In addition, there was a significant main effect of supplemental Cu level on the Cu content in feces, urine, serum, bile, kidney, liver, and lung samples (Table 3). Supplemental Cu at 20 mg/kg feed increased the Cu content of feces, urine, serum, and kidney samples over both the control and 5 mg/kg treatments. The Cu contents in bile and liver increased gradually with 5 and 20 mg/kg supplementation. Similarly, the lung had the highest Cu content with 20 mg/kg supplementation and the lowest with the control diet.

3.3. mRNA expression of genes

In the jejunal mucosa, the *CTR1* mRNA expression for supplemental Cu treatments was down-regulation compared to that of the control (Fig. 1A), and *CTR1* mRNA expression was higher at 20 m/kg level Cu-pro treatment compared to 20 mg/kg $CuSO_4$ treatment. The ATPase copper transporting alpha (*ATP7A*) gene expression was down-regulated ($P = 0.042$) in 20 mg/kg Cu treatments (Fig. 1B). *ASCT2* gene expression was up-regulated ($P = 0.007$) in 20 mg/kg Cu-Gly treatment (Fig. 1D). In addition,

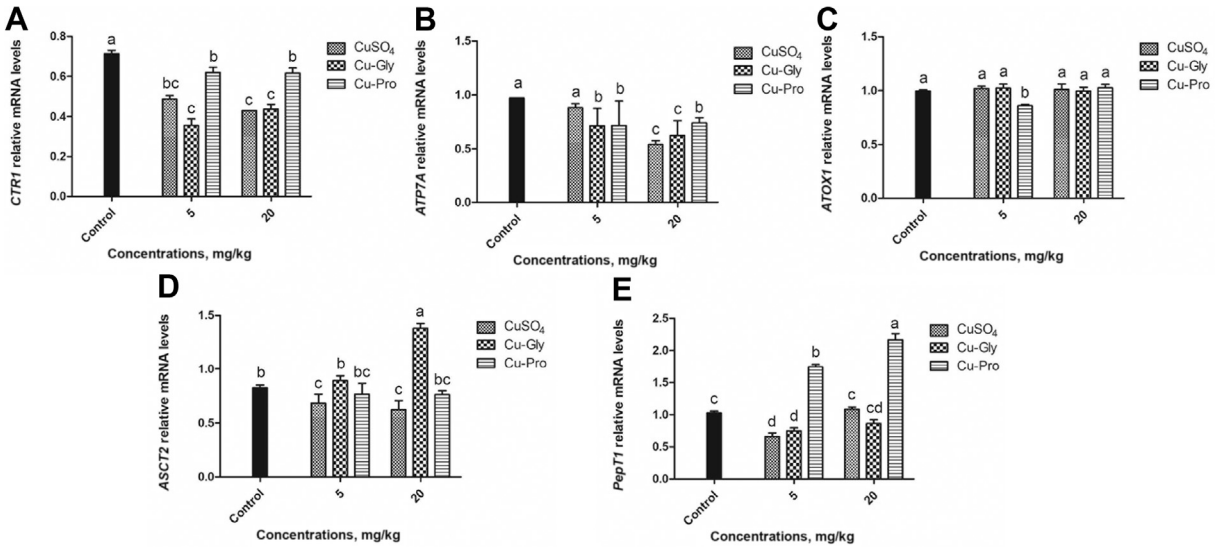

Fig. 1. Effects of different copper sources and levels on the mRNA expression of *CTR1*(A), *ATP7A*(B), *ATOX1*(C), *ASCT2*(D) and *PepT1*(E) genes in jejunal mucosa. $CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; *CTR1* = high affinity copper uptake protein 1; *ATP7A* = ATPase copper transporting alpha; *ATOX1* = Antioxidant 1 copper chaperone; *ASCT2* = lanine-serine-cysteine transporter, type-2; *PepT1* = peptide transporter 1. Data represent mean values ± standard error of the mean ($n = 6$). Significant differences between processing treatments are represented by different lowercase letters ($P < 0.05$).

PepT1 gene expression in Cu-Pro supplementation at both levels was higher ($P = 0.004$) than that of the control and other Cu-sources, and the expression level at 20 mg/kg Cu-Pro treatment was significantly higher than at 5 mg/kg Cu-Pro treatment (Fig. 1E).

In the liver, down-regulation ($P = 0.047$) of *CTR1* gene expression for all treatments compared to the control was observed, and the expression of *CTR1* gene was the lowest in 20 mg/kg Cu-Gly treatment (Fig. 2A). The *ATP7A* gene expression was down-regulated ($P = 0.043$) in all the other treatments compared to the control and 5 mg/kg $CuSO_4$ treatments (Fig. 2B). The ATPase copper transporting beta (*ATP7B*) gene expression in 20 mg/kg $CuSO_4$ and Cu-Gly treatments was up-regulated compared to that in control and 5 mg/kg treatments. Further, *ATP7B* gene expression in 20 mg/kg $CuSO_4$ was higher than both 20 mg/kg organic Cu treatments (Fig. 2C). *ATOX1* was up-regulated ($P = 0.047$) with all three 20 mg/kg treatments compared with the control and the 5 mg/kg treatments, regardless of Cu sources. *ASCT2* gene expression was up-regulated ($P = 0.038$) in Cu-Gly treatments, with the expression level at 20 mg/kg Cu-Gly treatment being significantly higher than that at 5 mg/kg Cu-Gly treatment (Fig. 2E).

3.4. Protein expression in jejunal mucosa

Compared to the control, the down-regulation ($P = 0.038$) of CTR1 protein expression in jejunal mucosa was observed in 5 mg/kg Cu-Gly and Cu-Pro treatments at and with all 3 Cu supplementation

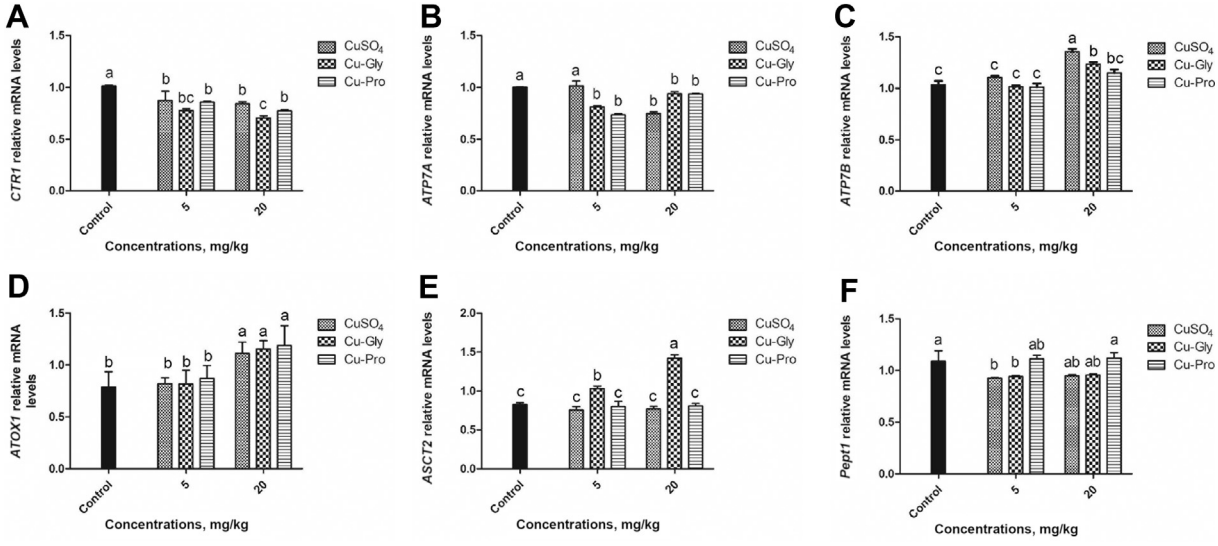

Fig. 2. Effects of different copper sources and levels on the mRNA Expression of *CTR1*(A), *ATP7A*(B), *ATP7B*(C), *ATOX1*(D), *ASCT2*(E) and *PepT1*(F) genes in liver. $CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; *CTR1* = high affinity copper uptake protein 1; *ATP7A* = ATPase copper transporting alpha; *ATOX1* = Antioxidant 1 copper chaperone; *ASCT2* = lanine-serine-cysteine transporter, type-2; *PepT1* = peptide transporter 1. Data represent mean values ± standard error of the mean ($n = 6$). Significant differences between processing treatments are represented by different lowercase letters ($P < 0.05$).

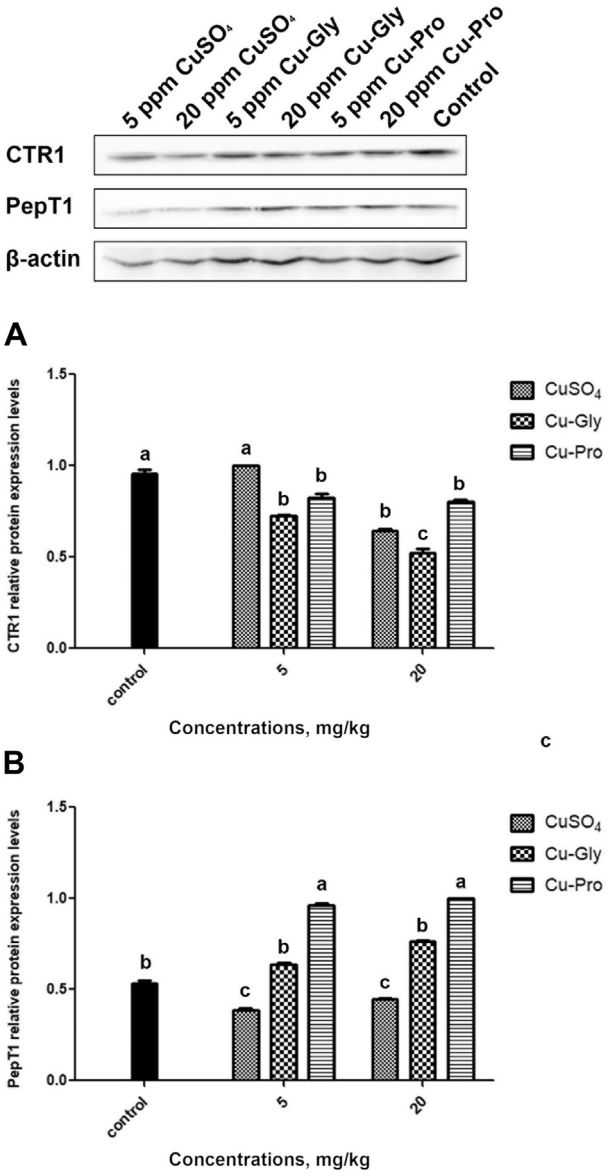

Fig. 3. Effects of different copper sources and levels on the protein expression for CTR1(A), and PepT1(B) in jejunal mucosa. $CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; CTR1 = high affinity copper uptake protein 1; PepT1 = peptide transporter 1. Data represent mean values ± standard error of the mean ($n = 6$). Significant differences between processing treatments are represented by different lowercase letters ($P < 0.05$).

forms at 20 mg/kg, where Cu-Gly was significantly lower than the other 2 forms (Fig. 3A). The protein expression of PepT1 (Fig. 3B) was significantly up-regulated at both Cu-Pro treatments ($P = 0.006$), and significantly down-regulated at both $CuSO_4$ treatments ($P = 0.046$).

3.5. Short chain fatty acids

Table 4 shows the results of the short chain fatty acids in colonic digesta. Lactic acid was elevated in 20 mg/kg Cu-Gly treatment compared to the control and 20 mg/kg $CuSO_4$ treatments, with Cu-Pro being intermediate. Acetate concentration of Cu-Gly treatment was significant higher than that of the other treatments. There were no statistical differences for the other SCFAs among the 4 treatments.

Table 4
Effects of different copper sources on short chain fatty acid concentrations in the colonic chyme of finishing pigs (mg/kg).

Item	Control[1]	20 mg/kg $CuSO_4$	20 mg/kg Cu-Gly	20 mg/kg Cu-Pro	SEM	*P*-value
Lactic acid	9.14[b]	5.77[b]	18.27[a]	12.42[ab]	1.345	<0.001
Acetate	625.44[b]	614.42[b]	677.04[a]	613.23[b]	20.50	0.022
Propionate	401.43	392.06	404.05	400.74	24.93	0.999
Formic acid	15.12	10.62	15.06	14.41	1.06	0.405
Isobutyrate	28.80	26.56	28.57	26.94	1.55	0.950
Butyrate	227.74	227.01	252.72	235.41	20.08	0.972
Valerate	53.32	50.63	57.96	50.85	2.20	0.652
Isovalerate	47.86	49.64	54.09	46.82	4.04	0.937

$CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; SEM = standard error of the mean.
[a,b]Means in the same row with different superscripts differ ($P < 0.05$, $n = 6$).
[1] Control, negative control diet without any copper addition.

3.6. Microflora community

The results of microbial community analysis for the 4 treatments show that the 16S rRNA gene sequencing generated a total of 679, 945 quality sequences with an average of 415.3 sequences per sample. There were statistical differences in the alpha diversity of colonic digesta microbial communities among the 4 treatments (Table 5). The observed richness (Sobs), the Chao1 estimator and the ACE estimator of the microbial community in $CuSO_4$ treatment significantly decreased ($P < 0.01$, $P = 0.072$, $P < 0.05$) in comparison to the control treatment and Cu-Pro treatment. The Shannon index of the microbial community in $CuSO_4$ treatment was lower ($P < 0.05$) compared with the control treatment. Furthermore, the observed richness (Sobs), the Chao1 estimator and the ACE estimator of the microbial community in Cu-Gly treatment tended to decrease ($P < 0.05$, $P = 0.082$, $P = 0.096$) compared with that in the control treatment. The observed richness (Sobs), Shannon index, and ACE estimator of the microbial community in $CuSO_4$ treatment significantly decreased ($P < 0.05$, $P < 0.05$, $P = 0.069$, respectively) in comparison to the Cu-Pro treatment. Meanwhile, the Simpson index of the microbial community for the $CuSO_4$ fed treatment tended to increase ($P = 0.092$) compared with the Cu-Pro treatment.

The relative abundances of bacteria in 3 phyla, 5 classes, 8 orders, 8 families and 22 genera were different among the 4 treatments. At the phylum level, a total of 6 phyla were detected (Fig. 4A). Firmicutes, Bacteroidetes, Spirochaetae and Actinobacteria were the dominant bacterial phyla and their total relative abundance accounted for 97% in all treatments. The control treatment had a lower relative abundance of Firmicutes ($P < 0.05$) compared with the Cu-Gly treatment (Fig. 4B). And the relative abundance of Bacteroidetes, and Spirochaetae in the control treatment tended to increase compared with the Cu-Gly treatment (Fig. 4B). At the family level, a total of 15 families were detected in 4 treatments (Fig. 4C). Muribaculaceae, Clostridiaceae_1, Lactobacillaceae, Peptostreptococcaceae, Ruminococcaceae, Spirochaetaceae, Lachnospiraceae, Streptococcaceae and Prevotellaceae were the dominant bacterial families. The control treatment had a higher relative abundance of Ruminococcaceae ($P < 0.05$) compared with the $CuSO_4$ treatment and Cu-Gly treatments. The relative abundance of Muribaculaceae, Christensenellaceae and Veillonellaceae treatment tended to increase in the control treatment compared with other treatments, especially the Cu-Gly treatment. In addition, the control treatment tended to have a lower relative abundance of Clostridiaceae_1, Peptostreptococcaceae and Lactobacillaceae compared with other treatments (Fig. 4D).

Table 5
Effects of different copper sources on the α-diversity[1] of microbial communities of finishing pigs in colonic digesta.

Item	Control[2]	20 mg/kg $CuSO_4$	20 mg/kg Cu-Gly	20 mg/kg Cu-Pro	*P*-value
Sobs	473.75[a]	369.25[c]	432.75[bc]	448.75[ab]	0.032
Shannon	3.43[a]	2.78[b]	3.16[ab]	3.29[a]	0.022
Simpson	0.11	0.19	0.12	0.10	0.092
ACE	568.87[a]	463.22[b]	526.18[a]	547.69[a]	0.041
Chao1	572.50	485.03	522.89	555.20	0.072
Coverage	0.996	0.996	0.996	0.996	0.275

$CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate; SEM = standard error of the mean.
[a,b,c] Means within the same row with different superscripts differ significantly ($P < 0.05$, $n = 6$).
[1] Alpha-diversity analysis for bacterial community determined by 16S rRNA gene sequencing.
[2] Control, negative control diet without any copper addition.

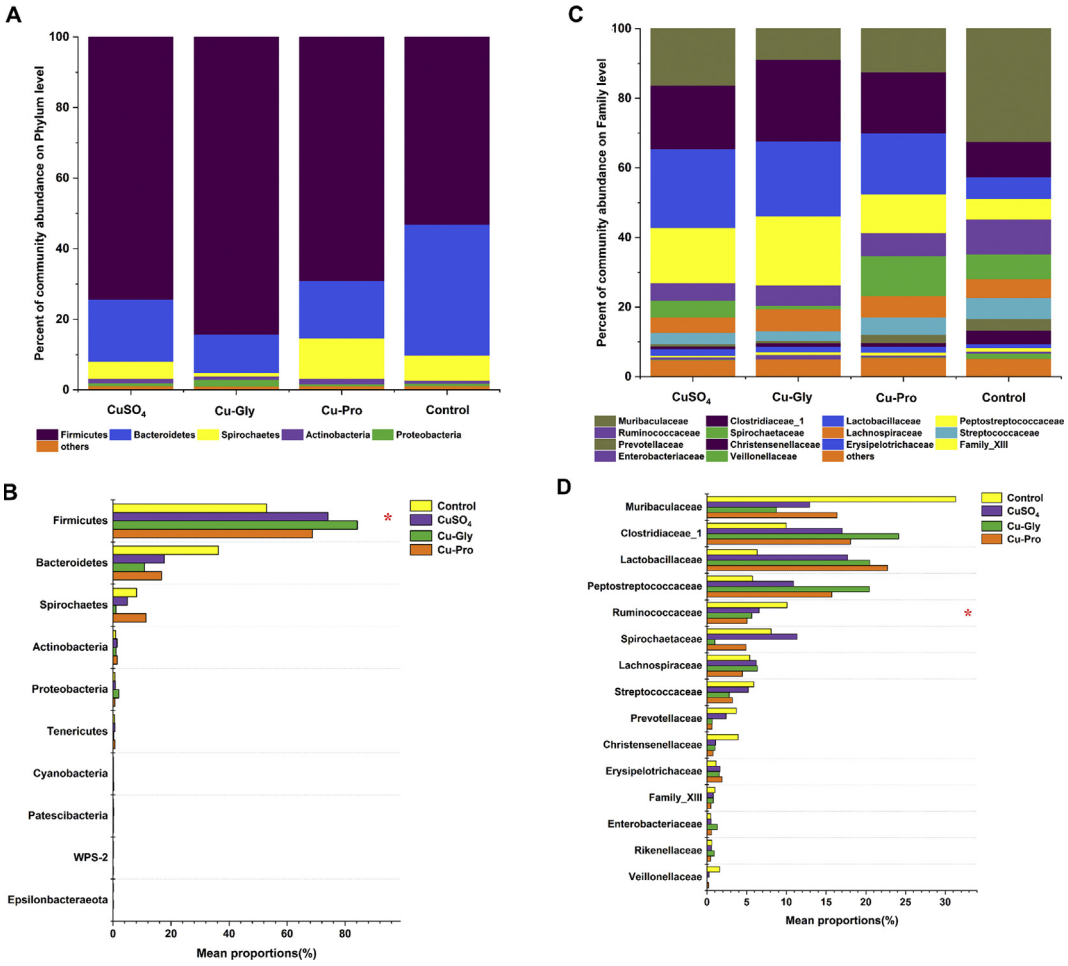

Fig. 4. Microbial community diversity in colonic digesta of finishing pigs on the phylum and family levels in colonic digesta ($n = 6$). (A) Microbial community bar plot on the phylum level with the relative abundance higher than 0.01%. (B) The bar plot was used to represent the dominant populations at the phylum levels by Kruskal–Wallis H test. * represented $P < 0.05$. (C) Microbial community bar plot on the family level with the relative abundance higher than 0.01%. (D) The bar plot was used to represent the dominant populations at the family levels by Kruskal–Wallis H test. * represented $P < 0.05$. $CuSO_4$ = copper sulfate; Cu-Gly = copper glycinate; Cu-Pro = copper proteinate. Control, negative control diet without any copper addition.

4. Discussion

Cu is generally used as growth stimulators in the pig industry. The effects of different Cu supplementations on growth performance and carcass characteristics are controversial. Most existing research has suggested that the growth-promoting effect of Cu mainly occurs in the early finishing stage and only at high doses of Cu. Hastad et al. (2004) found that pigs supplemented with increased Cu (50, 100 and 200 mg/kg) had improved growth performance of up to 61 kg and no effect observed thereafter. However, Zhao et al. (2014) reported that ADG and G:F tended to increase from 101 kg to 118 kg when Cu inclusion was increased from 6 to 170 mg/kg. In our study, ADG and G:F increased with addition of Cu at both 5 and 20 mg/kg in finishing pigs, but there were no significant differences in Cu sources and doses. However, converse previous reports (Carpenter et al., 2019) stated that growth performance is not affected by Cu source. Unexpectedly, in our study, ADG and G:F increased with addition of Cu at both 5 and 20 mg/kg in finishing pigs. Contradicting previous views (Zhou et al., 1994), higher feed intake was not the reason for promoting growth performance in this study as no intake differences were observed among Cu intake levels. The suggested growth-promoting effects of

supplementing weanling pigs with Cu has previously been linked to alterations in the composition of intestinal bacteria (Zhao et al., 2007). However, the development of intestinal bacterial populations in finishing pigs may be relatively mature and unlikely to be significantly altered, and subsequent testing has confirmed this. However, the suggested growth-promoting effects of supplementing weanling pigs with Cu has previously been linked to alterations in the composition of intestinal bacteria (Zhao et al., 2007). Combined with previous studies, the differences in results may be related to the dose of Cu and the growth period of pigs. Conversely, in contrast to previous reports, Carpenter et al. (2019) stated that growth performance is not affected by Cu source. In addition, the results show that the value of supplemental Cu, minimal growth performance and carcass characteristics changes were observed between 5 and 20 mg/kg of Cu supplementation.

Inclusion of high levels of dietary Cu in pigs can impair animal health and performance through accumulation of Cu in the animal's tissues while simultaneously contaminating the environment due to excessive Cu excretion. Zhai et al. (2006) reported that fecal Cu content increased with increasing dietary Cu levels. Our study drew a similar conclusion and additionally observed that use of organic Cu treatment at 20 mg/kg can significantly reduce the excretion of Cu, which was in accordance with previous research (Lin et al., 2020). Cu concentrations in the liver increased with increased Cu inclusion in the diet, and addition of Cu at 20 mg/kg demonstrated higher levels in the liver of organic Cu treatments than that in the inorganic Cu treatment. Our study results are consistent with previous studies that observed higher concentrations of Cu in the liver, kidney, brain, and heart, with lower concentrations found in the bones and muscles (Adams et al., 2019; Pena et al., 1999). In addition, sustained high Cu intake may lead to an excess Cu accumulation in the liver, which may affect the transportation and storage capacity of Cu (Sternlieb, 1980), hence leading to excess Cu leakage into the circulatory system (Floriańczyk, 2003). The present study found a link between serum Cu levels and Cu levels in organs, suggesting that feeding 20 mg/kg of supplementary Cu will exceed liver Cu thresholds in the fattening period.

Like the previous studies (Lin et al., 2020; Huang et al., 2015), biliary Cu concentrations had a similar change to liver Cu concentrations in the current study. Interestingly, considering that most of the Cu in the diet is not absorbed but is excreted directly through feces, the pigs in this study were sensitive to small changes in dietary Cu levels and Cu sources as evidenced by differences in the deposition and excretion of Cu. Mahoney et al. (1955) indicated that the excretion of endogenous Cu is mainly via the bile, thus the biliary Cu may have had similar responses to fecal Cu. For our results, we found that compared with the content of Cu in feed (397.39 mg), there was a significant enrichment of Cu in feces (427.37 mg). We hypothesise that the increased excretion in Cu from the pigs during fattening is potentially from endogenous Cu. This may be one of the reasons why adding a small amount of Cu during the fattening period can also cause serious Cu excretion. In combination with the fecal Cu content results, this study highlights that organic Cu may have a greater bioavailability to pigs. Organic Cu's properties may reduce the amount of supplementary Cu required in feed for pig production, and also reduce Cu wastage through lower excretion.

Cu transporters are critical tools for absorption, utilization, and maintenance of Cu homeostasis. The uptake of Cu by the intestine and liver is mainly mediated through CTR1, a Cu import transporter. When entering the cell, Cu can be transmitted to ATP7A and ATP7B through the chaperone protein ATOX1 in the intestine and liver, respectively. Huang et al. (2015) reported that the mRNA expression of *CTR1* and *ATP7A* was not affected by Cu concentration or Cu source in the liver or any intestinal section. However in our study, Cu import transporter *CTR1* and Cu export transporter *ATP7A* were down-regulated in 20 mg/kg treatments (except for 20 mg/kg $CuSO_4$ treatment) in the jejunum mucosa and liver when compared to the control, with similar results for CTR1 protein expression. The results indicated that the cells decreased mRNA expression of these 2 genes in order to regulate cellular Cu flux, thus maintaining Cu homeostasis.

ATOX1 is an important Cu chaperone protein that maintains Cu homeostasis by binding and transporting cytosolic Cu to ATPase proteins. ATOX1 is activated by the efflux of Cu from cells under conditions of elevated extracellular Cu concentrations (Pereira et al., 2016; Fontaine and Mercer, 2007). In our study, *ATOX1* mRNA expression was up-regulated in the liver for treatments fed 20 mg/kg diet with the same Cu source. The upregulation of *ATOX1* mRNA expression was also correlated to hepatic Cu concentrations, possibly because higher intracellular Cu content stimulated the mRNA expression of *ATOX1* (Doguer et al., 2018). This study indicated that both the level and source of added Cu could affect *ATP7B* expression in the liver, with *ATP7B* expression in 20 mg/kg treatments being higher than in 5 mg/kg treatments. Additionally, we found that Cu-Gly and Cu-Pro treatments can significantly down-regulate the mRNA expression of *ATP7B* compared with $CuSO_4$ treatment. Combined with previous findings, the regulation of *ATP7B* was contrary to liver Cu content and biliary Cu concentration. The role of *ATP7B* as a key Cu exporter may contribute to the change observed in liver Cu content.

The jejunal mucosa expression of *PepT1* and *ASCT2* were significantly up-regulated in the Cu-Pro treatment and Cu-Gly treated treatments. PepT1 protein expression was similarly significantly higher for both levels of Cu-pro treatments. Li et al. (2020) also found similar results in IPEC-J2 cells. Two key considerations have been drawn from the research findings here: firstly, that glycine or small peptides carried Cu into the cell by endocytosis; and secondly, Cu-Gly and Cu-Pro underwent dissociation before transport by *ASCT2* and *PepT1*. The dissociated glycine or small peptides from Cu transporters results in increased Cu utilization (Li et al., 2020; Ashida et al., 2002). Findings to date have not been able to discern the relationship between elevated Cu bioavailability and high mRNA expression of *ASCT2* or *PepT1*. Further research is required to determine if there is a link with the transporters of organic Cu.

Intestinal microbiota composition and diversity affects intestinal health (Blachier et al., 2017) and total tract digestion of nutrients through a range of physiological functions (Seo et al., 2015). In our experiment, there were statistical differences in the α-diversity indices (Sobs, ACE estimator, Simpson index, Chao and Shannon indexes) of the microbiota community in colonic digesta samples of pigs among the 4 Cu treatments. These results indicated that Cu supplementation shaped microbiota diversity of pigs. There are limited studies related to the microbiota community of finishing pigs. Shurson et al. (1990) found that a 283 mg/kg Cu diet indirectly affected growth performance by inhibiting the gut microbiota, and Miller et al. (1986) found that piglets receiving 250 mg/kg Cu had reduced total fecal bacteria by 60%. This study had similar results on the diversity of microbiota communities, with 20 mg/kg $CuSO_4$ demonstrating significantly reduced diversity and 20 mg/kg Cu-Gly tending to reduce diversity. There is no consensus on the effect of Cu on microbial diversity as other studies have found differing results. Perez et al. (2011) found that piglets receiving 250 mg/kg $CuSO_4$ or 100 mg/kg Cu-Gly had no change in diversity of microbiota communities. In our study, organic Cu treatments marginally reduced the diversity of microbiota communities compared with the control treatment. The inhibition effect of different Cu sources on bacteria may be related to the chemical structure of Cu and its dissociation

coefficient from the organic ligand. In general, if the gastrointestinal microbiota of piglets was in equilibrium, the dominant bacteria would be Lactobacillaceae, Peptostreptococcaceae, Streptococcaceae, Bifidobacteriaceae and Enterobacteriaceae (Alexandru et al., 2009; Maxwell et al., 2004; Fuller et al., 1978). However, in our study, the dominant bacteria in the colonic digesta of finishing pigs were Muribaculaceae, Clostridiaceae_1, Lactobacillaceae, Peptostreptococcaceae, Ruminococcaceae, Spirochaetaceae, Lachnospiraceae and Streptococcaceae, although the dominant bacteria in the small intestine was Lactobacillaceae. These differences may be related to different intestinal segments and pig developmental stage. In our study, the ratio of Firmicutes to Bacteroidetes in the control treatment was significantly lower than that in other treatments, especially Cu-Gly treatments. This is of interest as an increased ratio of Firmicutes to Bacteroidetes is an important occurrence marker of obesity (Mariat et al., 2009). However, Christensenellaceae can effectively prevent the body from getting fat (Waters et al., 2019). The relative abundance of Christensenellaceae in the control treatment pigs tended to be higher compared with the Cu treatments. Given that both intramuscular fat and marbling score increased with the addition of Cu and that the tenth rib fat depth ($P = 0.058$) tended to be higher when Cu was added, the effect of Cu-mediated changes in the microbiome on finishing efficiency and carcass composition in finishing pigs should be considered in future research. In addition, we found that finishing pigs receiving Cu supplementation tended to have lower relative abundance of Streptococcaceae. These findings of this study were in agreement with the results of Fuller et al. (1960) who reported that $CuSO_4$ in piglets' diets could significantly reduce the relative abundance of Streptococcaceae. Alternative studies showed that Lactobacillaceae were able to protect the host from pathogenic bacteria (Xian-Gang et al., 2008). However, Jensen found that pigs receiving a high Cu diet had reduced relative abundance of Lactobacillaceae (Jensen, 1998). In our study, the relative abundance of Lactobacillaceae in the control treatment tended to be lower than that in the Cu-Gly treatment. We also found that the change of lactic acid concentration was related to Lactobacillaceae. The results indicated that 20 mg/kg Cu may enhance the abundance of probiotics. In addition, it was previously shown that Clostridiaceae_1 and Peptostreptococcacea were associated with carbohydrate degradation and greater feed utilization capacity (Mao et al., 2015). In our study, the Cu-Gly treatment tended to have a higher relative abundance of Clostridiaceae_1 compared with other treatments. However, we couldn't connect this to carbohydrate digestion and feed efficiency using the data from the current study.

5. Conclusions

In this study, we analyzed differences among organic and inorganic Cu sources, regarding the amount of Cu deposited in tissues and assessed the potential reasons for the higher bioavailability of organic Cu. In the concentration range of this experiment, the addition of Cu was able to increase the daily gain and G:F of finishing pigs. Unlike inorganic Cu ($CuSO_4$) supplementation, use of organic Cu (Cu-Pro and Cu-Gly) significantly lowered Cu excretion, resulting in higher Cu retention. Moreover, the apparent metabolism of Cu was closely related to the transport carrier, though the transport mechanisms of organic Cu need to be further investigated. Finally, while both organic Cu and inorganic Cu have the same important effect on growth performance and characteristics, organic Cu sources were superior to inorganic Cu as they ameliorated Cu excretion and improved microflora abundance. Therefore, organic Cu supplementation may offer an effective alternative to inorganic Cu supplementation in swine production.

Author contributions

Wen Yang: Data curation, formal analysis, investigation, writing—original draft; preparation, writing—review and editing, project administration. **Li Runxian:** investigation, writing—original draft preparation. **Piao Xiangshu:** Supervision. **Lin Gang:** Conceptualization. **He Pingli:** funding acquisition, Supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.10.007.

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