

Article

Nano-Copper Supplementation Reduces Fecal Copper Excretion and Enhances Piglet Performance Under Heat Stress

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Abstract: This study aimed to evaluate the effects of dietary nano-copper supplementation on growth performance, nutrient digestibility, antioxidant status, inflammatory response, and intestinal barrier function in weanling pigs under heat stress conditions. Forty 20-day-old weaned weanling pigs (Yorkshire × Landrace × Duroc) weighing 6.49 ± 0.08 kg were randomly divided into five treatments with eight replicates each. The pre-feeding period was 2 days, followed by a 22-day experimental period. All groups were exposed to high heat conditions at 35 ± 1 °C. The control group received a basal diet, while the low copper sulfate (LC) group received a diet with 50 mg/kg of copper sulfate, the high copper sulfate (HC) group received a diet with 150 mg/kg of copper sulfate, the low nano-copper (LNC) group received a diet with 50 mg/kg of nano-copper oxide, and the high nano-copper (HNC) group received a diet with 150 mg/kg of nano-copper oxide. Compared to the basal group, pigs supplemented with copper (either CuSO₄ or nano-CuO) exhibited significantly higher average daily gain (ADG, $p < 0.048$) and feed intake (ADFI, $p = 0.005$), with the 50 mg/kg nano-copper group showing improved nutrient digestibility ($p < 0.05$) and intestinal morphology. Nano-copper supplementation significantly enhanced mucosal SOD activity ($p < 0.05$), reduced MDA levels ($p < 0.05$), and downregulated pro-inflammatory cytokines such as IL-1 β and IL-6 ($p < 0.05$). Notably, 50 mg/kg of nano-copper increased the apparent total tract digestibility (ATTD) of copper to 30.29%, significantly higher than the 16.55% observed in the 150 mg/kg CuSO₄ group ($p < 0.05$). Furthermore, fecal copper concentration was significantly reduced by 20.7% in the 50 mg/kg nano-copper group compared to copper sulfate ($p < 0.001$). In conclusion, nano-copper appears to be a promising alternative to copper sulfate for improving growth performance and reducing fecal copper concentrations in weanling pigs under heat stress conditions.

Keywords: nano-copper; copper pollution; weanling pigs; heat stress; inflammation; anti-oxidation



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1. Introduction

Copper, an indispensable trace element, serves as a critical cofactor for enzymes governing redox reactions (e.g., cytochrome c oxidase), energy metabolism (e.g., ATP7A/B), and antioxidant defense systems (e.g., Cu/Zn-SOD) in mammals [1–3]. In swine production, copper supplementation has been a cornerstone strategy for decades, with inorganic

forms like copper sulfate (CuSO_4) enhancing growth performance by 10–15% through antimicrobial effects and nutrient utilization [4,5]. However, a large proportion (approximately 70–90%) of dietary copper is excreted unmetabolized, which reduces utilization efficiency in animals and may increase copper load in the feces [6,7]. This is particularly concerning under intensive farming conditions where excessive trace mineral excretion can influence microbial balance and nutrient cycling [8,9]. Improving copper bioavailability is therefore critical not only for animal health and performance but also for minimizing resource inefficiency. Compounding this issue, heat stress—a pervasive challenge in tropical and subtropical pig farming regions—exacerbates copper wastage by impairing intestinal absorption efficiency by up to 30% [10]. For instance, studies in Southwest China, where summer temperatures routinely surpass 35 °C, demonstrate that heat-stressed pigs exhibit reduced villus height and increased intestinal permeability, further diminishing mineral retention [11,12].

The advent of nanotechnology offers a paradigm shift. Engineered nanoparticles, such as nano-copper oxide (nano-CuO), exhibit unique physicochemical properties, including high surface reactivity and enhanced cellular uptake, which can improve bioavailability by 40–60% compared to conventional CuSO_4 [13,14]. In poultry trials, nano-CuO supplementation at 50 mg/kg reduced fecal copper excretion by 35% while maintaining growth performance, highlighting its dual economic and environmental benefits [15]. Similarly, rodent models reveal that nano-CuO enhances antioxidant enzyme activity (e.g., SOD and CAT) under oxidative stress, suggesting potential applications in mitigating heat-induced metabolic dysfunction [4]. Despite these advances, research on nano-copper in swine remains nascent, particularly under heat stress, a critical oversight given that over 60% of global pork production occurs in climate-vulnerable regions [16]. Current literature predominantly focuses on growth metrics, neglecting systemic evaluations of gut health, inflammatory responses, and long-term environmental impacts [7,17]. For example, a 2022 meta-analysis identified only three studies examining nano-copper's effects on swine intestinal morphology, none of which addressed thermal stress [5].

Although copper is widely used in pig nutrition, the dose-dependent effects and environmental implications of nano-copper supplementation under heat stress conditions remain inadequately explored. Against this backdrop, this study posits that nano-CuO supplementation can concurrently alleviate heat stress-induced growth suppression in weanling pigs and reduce environmental copper pollution through optimized bioavailability. We hypothesized that nano-CuO would outperform traditional CuSO_4 in weanling pigs, based on prior studies indicating that nano-copper has higher bioavailability and more favorable effects on antioxidant capacity and intestinal health compared to conventional copper sources [18,19]. To test this, we conducted a controlled trial comparing five dietary treatments under simulated heat stress (35 °C), assessing parameters spanning growth performance, nutrient utilization, oxidative stress biomarkers, and fecal copper dynamics. Our findings not only address critical gaps in sustainable swine nutrition but also align with global initiatives such as the FAO's Climate-Smart Agriculture framework, which emphasizes resource-efficient livestock systems [20]. By integrating physiological, environmental, and technological perspectives, this work provides a blueprint for reconciling productivity gains with ecological stewardship in intensive animal agriculture.

2. Materials and Methods

2.1. Animal, Diets, and Experimental Design

All animal protocols and procedures were approved by the Animal Care and Use Committee at Sichuan Agricultural University, Chengdu, Sichuan, People's Republic of China (No.20210102). The experiment was conducted at the Animal Experiment Center

of Sichuan Agricultural University in strict accordance with institutional guidelines for the care and use of laboratory animals. The research question, key design features, and analysis plan were developed before the experiment, following standard methodologies in animal studies.

Based on initial average body weight (6.49 ± 0.08 kg), 40 male weanling pigs at 23 days of age (Yorkshire $\times$ Landrace $\times$ Duroc, weaned at 20 days of age, followed by a 2-day adaptation period to minimize weaning stress) were randomly allotted to five treatments as follows:

- (1) Basal diet without copper supplementation (Basal);
- (2) Basal diet + 50 mg/kg of Cu as copper sulfate (LC);
- (3) Basal diet + 150 mg/kg of Cu as copper sulfate (HC);
- (4) Basal diet + 50 mg/kg of Cu as nano-copper oxide (LNC);
- (5) Basal diet + 150 mg/kg of Cu as nano-copper oxide (HNC).

Randomization was performed in Microsoft Excel by sorting pigs within body weight strata and then assigning treatments using random number generation to ensure balanced distribution across groups.

L-lysine and L-threonine were obtained from Daqi Biotechnology Co., Ltd. (Yongning, China). DL-methionine was provided by CJ CheilJedang Corp. (Seoul, Republic of Korea). Vitamin premix components were supplied by DSM Nutritional Products (Rovimix® series, Kaiseraugst, Switzerland). Mineral premix additives were purchased from Chongwei Biotechnology Co., Ltd. (Shanghai, China). CuO nanoparticle purity was 99.9%, and particle size was 40 nm. The experimental diet exceeded the nutrient requirements as suggested by NRC and is presented in Table 1.

Table 1. Composition and nutrient levels of basal diets (as-fed basis, %).

Ingredients	%	Nutrient Level ¹	Content
Corn	56.8	Digestible energy (MJ/kg)	14.55
Soybean meal	17.9	Crude protein, %	21.55
Extruded soybean	3	Calcium, %	0.83
Fish meal	6.6	Total phosphorus, %	0.70
Whey powder	9.1	Available phosphorus, %	0.48
Soybean oil	2.95	Lysine, %	1.39
Sucrose	1.2	Methionine, %	0.45
CaCO ₃ (Ca $\geq$ 94.0%)	0.55	Cysteine + Methionine, %	0.68
CaHPO ₄ (P $\geq$ 16.5%)	0.6	Threonine, %	0.81
L-lysine-HCl ($\geq$ 78.8%, dry basis)	0.6	Tryptophan, %	0.17
DL-Methionine ($\geq$ 99.0%, dry basis)	0.15	Cu, (mg/kg)	7.30
L-Threonine ($\geq$ 98.5%, dry basis)	0.2		
NaCl ($\geq$ 99.1%)	0.05		
Vitamin-mineral premix ²	0.3		
Total	100		

¹ Nutrient levels were calculated values. ² Diets were supplemented with supplies following a per kg diet: Vit. A, 7500 IU; Vit. D3, 2500 IU; Vit E, 20 IU; Vit K, 2.5 mg; Riboflavin VitB2, 6.25 mg; d-pantothenic acid d-pantothenic acid VitB5, 12.5 mg; Niacin VitB3, 25 mg; Vit B12, 0.3 mg; Biotin, 0.5 mg; VitB1, 2.5 mg; Folic acid, 1.25 mg; Pyridoxine hydrochloride VitB6, 3 mg; I, 0.14 mg; Mn, 4 mg; Fe, 90 mg; Zn, 90 mg; Se, 0.3 mg.

Pigs were housed in a controlled climate room for a 22-day experimental period. The ambient temperature was maintained at 35 ± 1 °C during the daytime (08:00–20:00) to simulate typical summer heat stress conditions in Southwest China, and reduced to 28 ± 1 °C

during the nighttime (20:00–08:00) to reflect natural diurnal temperature variation. All animals were fed ad libitum with free access to water and feed.

2.2. Animal Performances and Sampling

The body weight of pigs and feed intake were recorded individually every week to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (F:G). Fecal scores were recorded every day to calculate the diarrhea index and diarrhea rate. The fecal score was determined as follows: 0, firm feces; 1, soft and formed feces; 2, soft and unformed feces; 3, fluid feces; 4, watery feces. Pigs with a fecal score greater than 1 were considered to have diarrhea.

Diarrhea index and diarrhea rate calculation equations:

$$\text{Diarrhea index} = (\text{Total fecal score}) / (\text{Total days})$$

$$\text{Diarrhea rate (\%)} = (\text{The number of pig with diarrhea} \times 100) / (\text{Total number of pig} \times \text{Days of each phase})$$

On day 22, blood samples were collected from the anterior vena cava of all pigs and allowed to clot at room temperature for 30 min before centrifugation at $3000 \times g$ for 10 min to obtain serum. The serum samples were stored at $-20\text{ }^{\circ}\text{C}$ for subsequent analysis of inflammatory cytokines, antioxidant indices, and biochemical parameters. Two hours after blood collection, all pigs were euthanized via intramuscular injection of Zoletil[®] (tiletamine–zolazepam combination, 10–15 mg/kg body weight), which induced deep anesthesia followed by euthanasia. The pigs were weighed and dissected to collect the liver and spleen. The weights of these organs were recorded to calculate visceral indices as a percentage of body weight (organ weight/body weight $\times 100$). The mid-jejunum and mid-ileum segments were collected and fixed in 4% paraformaldehyde for histomorphological examination. The mucosa of the mid-ileum was scraped, snap-frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ for gene expression and antioxidant enzyme activity analysis.

2.3. Measurement of Cu Concentration in Feces and Feed

A total of approximately 2 g of each sample of feces and feed was taken and dried at $105\text{ }^{\circ}\text{C}$ for 4 h. From each dried sample, precisely 0.2 g was then taken to measure the Cu concentration. The concentration of Cu was determined using a contraAA[®]700 spectrometer (Analytik Jena, Jena, Germany).

2.4. Measurement of Cu Concentration in Serum

Serum copper concentration was determined using a commercial colorimetric assay kit (Product No. E010-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), based on the complexometric method. Briefly, under acidic conditions, Cu^{2+} was released from ceruloplasmin and albumin, and subsequently reduced to Cu^{+} by ascorbic acid. The Cu^{+} then reacted with 3,5-dibromo-PAESA to form a blue complex, the absorbance of which was measured at 600 nm with a reference wavelength of 700 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Measurement of Nutrient Digestibility

To determine the apparent total tract digestibility (ATTD), 0.3% chromium oxide was added to each diet. Fecal samples were collected during the last 5 days to determine the ATTD of dry matter (DM), crude protein (CP), crude fat (EE), calcium (Ca), and total phosphorus (TP). The mixed fecal samples were then dried in a forced-air oven at $65\text{ }^{\circ}\text{C}$ for 48 h, followed by air-drying for an additional 24 h. The dried samples were then ground using a Wiley Mill (Thomas Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA).

equipped with a 1 mm screen to facilitate chemical analysis. Dry matter, crude protein, crude fat, calcium, and total phosphorus were determined in the feed and feces (AOAC, 2012). The ATTD was calculated using the following equation:

$$\text{ATTD (\%)} = 100 - [(\text{feed Cr} \times \text{nutrient feces}) / (\text{fecal Cr} \times \text{nutrient diet})] \times 100.$$

2.6. Cytokines Measurements

Ileal mucosa samples were homogenized with 1:9 (*w/v*) saline at ice-cold temperature and centrifuged to prepare mucosa homogenates. The protein concentration of mucosa homogenates was measured using a BCA assay with the BCA Protein Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA). Interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interferon- γ (IFN- γ), transforming growth factor- β (TGF- β), tumor necrosis factor- α (TNF- α), and heat shock protein 70 (HSP70) in serum and ileal mucosa were detected using commercially available swine ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China). The results of the ileal mucosa were normalized to the protein concentration in each sample.

2.7. Measurements of Antioxidant-Related Enzymes and Metabolites

The activities of total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), and concentration of malondialdehyde (MDA) were measured with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The results of the ileal mucosa were normalized to the protein concentration in each sample.

2.8. Analysis of Jejunal and Ileal Morphology

Segments measuring 2 cm in length were collected from the jejunum (80 cm from the duodenojejunal flexure) and ileum (about 15 cm from the ileocecal valve) within 10 min post-execution. The samples were fixed in paraformaldehyde, dehydrated using a graded series of ethanol, and embedded in paraffin. Cross sections measuring 5 microns in size were cut, dehydrated, and stained with hematoxylin and eosin (HE). Villus length and crypt depth were determined for each section using an optical binocular microscope (Olympus BX43, Olympus Optical Co. Ltd., Tokyo, Japan) and Image-Pro Plus software (Version 6.0). The villus height to crypt depth ratio (V:C) was calculated.

2.9. Quantitative Real-Time PCR (RT-qPCR) Analysis

Total RNA was extracted from the ileum mucosa using RNAiso Plus (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and integrity was verified by 1% agarose gel electrophoresis.

Genomic DNA was removed, and complementary DNA (cDNA) was synthesized using the PrimeScriptTM FAST RT Reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan), following the manufacturer's protocol. The synthesized cDNA was diluted twofold with EASY Dilution II buffer prior to analysis.

Quantitative real-time PCR (RT-qPCR) was performed using the QuantStudio[®] 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and TB Green[®] Premix Ex TaqTM II (Takara Bio, Otsu, Japan). Each 10 μ L reaction mixture contained 5 μ L of 2 $\times$ TB Green Premix, 0.2 μ M each of forward and reverse primers, $\leq$ 1.0 μ L of cDNA template, and nuclease-free water to reach the final volume. Thermal cycling conditions were as follows: initial denaturation at 95 $^{\circ}$ C for 30 s, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 5 s and annealing/extension at 60 $^{\circ}$ C for 10 s. A melt curve analysis was conducted at the end of amplification to confirm product specificity.

Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH as the internal reference gene. All primers were designed using NCBI Primer-BLAST based on *Sus scrofa* mRNA sequences and synthesized by Sangon Biotech (Shanghai, China). Primer sequences are listed in Table 2.

Table 2. Primer sequences, amplicon sizes, GenBank accession numbers, and amplification efficiencies used for RT-qPCR analysis.

Gene	Primer Sequence (5'-3')	Product Size (bp)	Accession No.	Amplification Efficiency (%)
Occludin	Forward, CTACTCGTCCAACGGGAAAG Reverse, ACGCCTCCAAGTTACCACTG	158	XM_005672525.3	97.2
Claudin-1	Forward, TTTCCTCAATACAGGAGGGAAGC Reverse, CCCTCTCCCCACATTCGAG	81	NM_001244539.1	95.8
ZO-1	Forward, CAGCCCCCGTACATGGAGA Reverse, GCGCAGACGGTGTTCATAGTT	114	XM_005659811	96.4
TGF- β 1	Forward, TGACCCGCAGAGAGGCTAT Reverse, CGGCCAGAATTGAACCCGT	106	NM_214015.2	94.3
TGF- β 2	Forward, GGATCTTGGGTGGAATGGA Reverse, GGCACAGAAGTTGGCATTGT	58	XM_021064298.1	95.5
HO-1	Forward, AGCTGTTTCTGAGCCTCCAA Reverse, CAAGACGGAAACACGAGACA	130	NM_001004027.1	96.9
Keap-1	Forward, GAGAGGTATGAACCCGAGCG Reverse, ACACTCTGCTGAGTTGAGGC	147	XM_005654811.3	97.5
NQO1	Forward, TGTAAGCCGGGAAAGGTGT Reverse, CCATTGAGGAGTTGGGTGCT	132	NM_001159613.1	96.0
NRF2	Forward, GCCCCTGGAAGCGTTAAAC Reverse, GGACTGTATCCCCAGAAAGTTGT	67	XM_021075133.1	96.7
GPX4	Forward, TGTGTGAATGGGGACGATGC Reverse, CTTCACCACACAGCCGTTCT	135	NM_214407.1	97.0
GPX1	Forward, GGCGGCGGGTTCGA Reverse, CGCCATTACCTCACACTTCT	55	NM_214201.1	97.2
CAT	Forward, AGCTTTGCCCTTGACAAAC Reverse, ACATCCTGAACAAGAAGGGGC	119	XM_021081498.1	95.8
SOD1	Forward, AGGGCACCATCTACTTCGAG Reverse, GATCACCTTCAGCCAGTCCT	81	NM_001190422.1	96.4
SOD2	Forward, CTGGACAAATCTGAGCCCTA Reverse, TTGAAACCGAGCCAACCC	156	NM_214127.2	98.0
TNF- α	Forward, CGTCGCCCACGTTGTAGCCAAT Reverse, GCCCATCTGTGCGCACCAACC	128	NM_214022.1	95.5
IL-1 β	Forward, CCAAAGAGGGACATGGAGAA Reverse, GGGCTTTTGTCTGCTTGAG	123	XM_021085847.1	96.9
IL-6	Forward, TGGCTACTGCCTTCCCTACC Reverse, CACACATCTCCTTCTCATTGC	153	NM_214399.1	97.5
IL-10	Forward, CGGCGCTGTCATCAATTTCTG Reverse, CCCCTCTCTTGAGCTTGCTA	89	NM_214041.1	96.0
IFN- γ	Forward, ACCAGGCCATTCAAAGGAGC Reverse, CGAAGTCATTCAAGTTCCAGAG	90	NM_213948.1	96.7
Bax	Forward, AAGCGCATTGGAGATGAATC Reverse, TGCCGTCAGCAAACATTC	121	XM_013998624.2	97.0
Bcl-2	Forward, TGCCTTTGTGGAGCTGIATG Reverse, GCCCGTGGACTTCACTTATG	144	XM_021099593.1	97.2
BAD	Forward, GAGTCGCCACAGCTCTTACC Reverse, GCGAGGAAGTCCCTTCTTGA	187	XM_021082883.1	95.8

Table 2. Cont.

Gene	Primer Sequence (5'-3')	Product Size (bp)	Accession No.	Amplification Efficiency (%)
Caspase-3	Forward, GTGGGACTGAAGATGACA Reverse, ACCCGAGTAAGAATGTG	190	NM_214131.1	96.4
Caspase-8	Forward, ATGTCGGACTGTCTGGGAGA Reverse, GTATCCCCGAGGCTTGCTTT	84	XM_021074714.1	98.4
Caspase-9	Forward, AATGCCGATTGCGTTACGT Reverse, CATTTGCTTGGCAGTCAGGTT	195	XM_003127618.4	95.5
HSP70	Forward, GCCCTGAATCCGCAGAATA Reverse, TCCCCACGGTAGGAAACG	152	NM_001123127.1	96.9
GAPDH	Forward, GGGCATGAACCATGAGAAGT Reverse, TGTGGTCATGAGTCCTTCCA	133	XM_021091114.1	99.5

2.10. Statistical Analysis

All data were analyzed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA). Prior to analysis, data were tested for normality and homogeneity of variance using the Shapiro–Wilk test and Levene’s test, respectively. Data are presented as mean $\pm$ standard error of the mean (SEM). Differences among treatments were evaluated using one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test for post hoc analysis. In cases where the data did not meet the assumptions for parametric analysis, a non-parametric Kruskal–Wallis test was performed.

The effects of different copper sources and concentrations on growth performance, nutrient digestibility, antioxidant enzyme activities, cytokine levels, and intestinal morphology were analyzed using the following model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} represents the observed value, μ is the overall mean, T_i is the fixed effect of the treatment, and e_{ij} is the residual error.

Differences were considered statistically significant at $p < 0.05$, while a trend was indicated when $0.05 \leq p < 0.10$.

3. Results

3.1. Growth Performance

Copper supplementation significantly improved feed intake (ADFI) and average daily gain (ADG) compared to the basal group ($p < 0.01$ for ADFI, $p < 0.05$ for ADG), indicating a positive impact on growth performance under heat stress conditions (Table 3). However, the feed-to-gain ratio (F:G, $p > 0.05$) and overall diarrhea rate ($p > 0.05$) remained statistically similar across treatment groups, suggesting that the benefits primarily reflect improved nutrient utilization rather than feed efficiency.

The liver index of the LNC group was significantly higher than that of the other groups (Table 4). However, the results indicated no significant changes in the concentrations of the AST and ALT enzymes and BUN in the serum of different treatment groups (Figure 1).

Table 3. Effect of nano-copper and copper sulfate on the growth performance and diarrhea of piglets under heat stress.

Parameter	Treatment ¹					<i>p</i> Value ²
	Basal	LC	HC	LNC	HNC	
ADFI (g)	200.97 ± 18.65 ^b	274.3 ± 15.44 ^a	284.5 ± 13.98 ^a	242.58 ± 12.65 ^{ab}	241.6 ± 15.23 ^{ab}	0.005
ADG (g)	133.41 ± 18.71 ^b	180.17 ± 13.88 ^a	194.46 ± 5.93 ^a	163.5 ± 13.4 ^{ab}	174.11 ± 13.52 ^{ab}	0.048
F:G	1.72 ± 0.35	1.54 ± 0.06	1.41 ± 0.03	1.52 ± 0.1	1.45 ± 0.05	0.793
Diarrhea Index	0.47 ± 0.05 ^a	0.27 ± 0.07 ^b	0.26 ± 0.06 ^b	0.27 ± 0.05 ^b	0.26 ± 0.02 ^b	0.047
Diarrhea Rate	1.64 ± 0.08 ^a	1.31 ± 0.12 ^b	1.35 ± 0.1 ^b	1.36 ± 0.09 ^b	1.34 ± 0.04 ^b	0.09

ADFI = average daily feed intake, ADG = average daily gain, F:G = feed-to-gain ratio. Data are presented as mean ± SEM, *n* = 8. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. ¹ Basal, basal diet with heat stress; LC, low dose of CuSO₄, 50 mg/kg CuSO₄ in basal diet with heat stress; HC, high dose of CuSO₄, 150 mg/kg CuSO₄ in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. ² *p* value represents the significance level of difference between different treatments (basal, LC, HC, LNC, HNC) with heat stress. ^{a,b} Different superscript letters in one row indicate significantly different values (*p* < 0.05).

Table 4. Effect of nano-copper and copper sulfate on the organ indices of piglets under heat stress.

Parameter	Treatment ¹					<i>p</i> Value ²
	Basal	LC	HC	LNC	HNC	
Liver index	29.38 ± 1.59 ^{ab}	29.02 ± 1.02 ^{ab}	25.97 ± 0.95 ^b	32.22 ± 1.27 ^a	27.3 ± 0.86 ^b	0.026
Spleen index	1.14 ± 0.05	1.29 ± 0.08	1.38 ± 0.11	1.28 ± 0.05	1.41 ± 0.11	0.163

Data are presented as mean ± SEM, *n* = 8. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. ¹ Basal, basal diet with heat stress; LC, low dose of CuSO₄, 50 mg/kg CuSO₄ in basal diet with heat stress; HC, high dose of CuSO₄, 150 mg/kg CuSO₄ in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. ² *p* value represents the significance level of difference between different treatments (basal, LC, HC, LNC, HNC) with heat stress. ^{a,b} Different superscript letters in one row indicate significantly different values (*p* < 0.05).

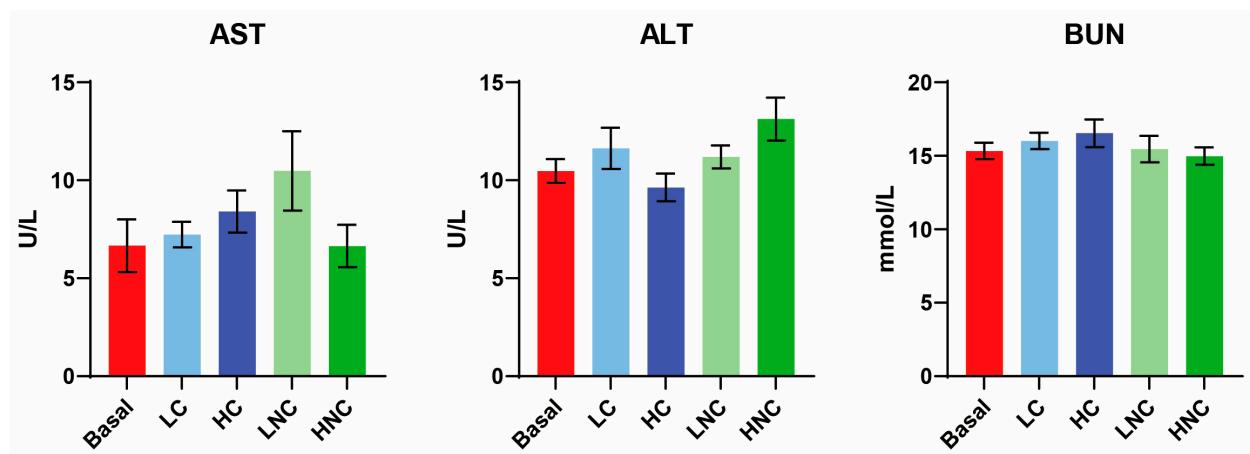


Figure 1. The effect of nano-copper and copper sulfate on AST, ALT, and BUN of piglets under heat stress. Data are presented as mean ± SEM, *n* = 8. Statistical analysis was performed using a one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. Basal, basal diet with heat stress; LC, low dose of CuSO₄, 50 mg/kg CuSO₄ in basal diet with heat stress; HC, high dose of CuSO₄, 150 mg/kg CuSO₄ in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, BUN = blood urea nitrogen.

3.2. Nutrient Digestibility

Compared to the basal group, the LC and LNC treatments (50 mg/kg of copper sulfate and 50 mg/kg of nano-copper, respectively) significantly improved the apparent total tract digestibility (ATTD) of dry matter (DM), crude protein (CP), and crude fat (CF) ($p < 0.05$, Table 5). However, the HC and HNC treatments (150 mg/kg of copper sulfate and 150 mg/kg of nano-copper) did not result in further improvement and instead showed a numerical or significant reduction in nutrient digestibility when compared with the low-dose treatments. There were no significant differences between LC and LNC or between HC and HNC for any of the measured digestibility parameters ($p > 0.05$), indicating that copper source (sulfate vs. nano-copper) did not affect nutrient digestibility at the same dose level. Additionally, total phosphorus digestibility was significantly decreased in both the LNC and HC groups when compared to the basal group ($p < 0.05$), suggesting a possible adverse effect of these treatments on phosphorus utilization.

Table 5. Effects of nano-copper and copper sulfate on nutrient digestibility in piglets under heat stress.

Parameter	Treatment ¹					<i>p</i> Value ²
	Basal	LC	HC	LNC	HNC	
DM (%)	80.92 ± 0.42 ^c	85.07 ± 0.56 ^{ab}	84.13 ± 0.78 ^{ab}	85.33 ± 0.41 ^a	82.9 ± 1.62 ^{bc}	0.04
CP (%)	71.16 ± 1.34 ^b	78.25 ± 1.41 ^a	76.98 ± 1.35 ^a	79.25 ± 1.28 ^a	76.4 ± 3.08 ^a	0.021
EE (%)	61.15 ± 3.38 ^c	78.76 ± 1.86 ^a	71.14 ± 1.87 ^{ab}	71.49 ± 1.2 ^{ab}	68.99 ± 5.14 ^{bc}	0.003
Ca (%)	57.46 ± 3.89	62.02 ± 2.05	65.23 ± 1.16	59.85 ± 7.46	54.9 ± 6.19	0.512
TP (%)	47.86 ± 4.31 ^a	50.48 ± 1.69 ^a	45.91 ± 0.91 ^{ab}	40.56 ± 1.08 ^b	40.66 ± 2.04 ^b	0.006

DM = dry matter, CP = crude protein, EE = ether extract, Ca = calcium, TP = total phosphorus. Data are presented as mean ± SEM, $n = 8$. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. ¹ Basal, basal diet with heat stress; LC, low dose of CuSO₄, 50 mg/kg CuSO₄ in basal diet with heat stress; HC, high dose of CuSO₄, 150 mg/kg CuSO₄ in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. ² *p* value represents the significance level of difference between different treatments (basal, LC, HC, LNC, HNC) with heat stress. ^{a-c} Different superscript letters in one row indicate significantly different values ($p < 0.05$).

3.3. Concentration in Serum, Feed, and Feces and Digestibility of Cu

The copper content in the basal group feces was 65.99 ± 4.572 ppm. For the addition of 50 mg/kg, the copper content in feces with copper sulfate was 410.4 ± 24.51 ppm, while the nano-copper group was 325.44 ± 30.846 ppm, resulting in a 20.7% reduction in copper emissions ($p < 0.001$). For the addition of 150 mg/kg, the copper content in feces was 890.13 ± 52.47 ppm in the copper sulfate group and 841.5 ± 24.39 ppm in the nano-copper group, with no statistically significant difference between the two treatments. Additionally, adding nano-copper can increase the copper digestibility.

Serum copper concentration was also significantly affected by copper supplements ($p = 0.006$). The highest serum copper level was observed in the HNC group (19.31 ± 1.13 µmol/L), which was significantly higher than the basal group (13.94 ± 0.58 µmol/L) and slightly higher than the HC group (17.21 ± 0.80 µmol/L), indicating that nano-copper may result in greater copper absorption or retention compared to copper sulfate at the same dose. At 50 mg/kg, both LC (14.92 ± 1.47 µmol/L) and LNC (15.41 ± 0.93 µmol/L) groups showed moderate increases with no significant difference.

In the control group without added copper, fecal copper excretion reflected endogenous copper loss. At a supplementation level of 50 mg/kg of copper sulfate, no significant improvement in copper retention was observed, and the apparent total tract digestibility (ATTD) of copper remained negative ($p > 0.05$ vs. control). Supplementation with 150 mg/kg of copper sulfate increased the ATTD to 16.55% ($p < 0.05$ vs. control). In contrast, 50 mg/kg of nano-copper significantly improved copper digestibility, reaching an ATTD of

30.29% ($p < 0.01$ vs. control; $p < 0.05$ vs. 150 mg/kg of copper sulfate). This value was also significantly higher than that of 150 mg/kg of nano-copper ($p < 0.05$), indicating a dose- and source-dependent effect on copper digestibility in weanling pigs (Table 6).

Table 6. Effect of nano-copper and copper sulfate on copper digestibility and serum copper concentration in piglets under heat stress.

Parameter	Treatment ¹					<i>p</i> Value ²
	Basal	LC	HC	LNC	HNC	
Cu Serum (μmmol/L)	13.94 ± 0.58 ^c	14.92 ± 1.47 ^{bc}	17.21 ± 0.8 ^{ab}	15.41 ± 0.93 ^{bc}	19.31 ± 1.13 ^a	0.006
Cu Feed (mg/kg)	9.24	53.46	169.94	65.44	150.72	
Cu Feces (ppm)	65.99 ± 4.57 ^c	410.4 ± 24.51 ^b	890.13 ± 52.47 ^a	325.44 ± 30.84 ^b	841.5 ± 24.38 ^a	<0.001
ATTD	−10.47 ± 4.78 ^c	−5.73 ± 7.47 ^c	16.55 ± 4.05 ^{ab}	30.29 ± 6.61 ^a	12.87 ± 1.95 ^b	<0.001
STTD	0 ± 4.78 ^c	4.75 ± 7.47 ^c	27.02 ± 4.05 ^{bc}	40.77 ± 6.61 ^a	23.34 ± 1.95 ^b	<0.001

ATTD = apparent total tract digestibility, STTD = standard total tract digestibility. Data are presented as mean ± SEM, $n = 8$. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. ¹ Basal, basal diet with heat stress; LC, low dose of CuSO₄, 50 mg/kg CuSO₄ in basal diet with heat stress; HC, high dose of CuSO₄, 150 mg/kg CuSO₄ in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. ² *p* value represents the significance level of difference between different treatments (basal, LC, HC, LNC, HNC) with heat stress. ^{a-c} Different superscript letters in one row indicate significantly different values ($p < 0.05$).

3.4. Intestinal Health

Two doses of different copper formulations were found to improve the morphology of the jejunum and ileum (Figure 2A,B). In comparison to the basal group, all copper-treated groups showed a decrease in crypt depth in the jejunum, with the 50 mg/kg dose of nano-copper significantly increasing the villus-to-crypt ratio (V:C) (Figure 2A). Both doses of nano-copper increased villus height while decreasing crypt depth in the ileum, resulting in a higher V:C compared to the basal group. However, 150 mg/kg of copper sulfate significantly decreased villus height and V:C in the ileum compared to the basal group, indicating a damaging effect of high-dose copper sulfate on the ileum (Figure 2B).

A dosage of 150 mg/kg of nano-copper significantly upregulated the mRNA expression of ZO-1, one of the key tight junction proteins, suggesting a potential enhancement of epithelial barrier integrity under heat stress conditions (Figure 2C). All copper treatments led to a significant decrease in the mRNA expression of Bax, with the Bcl-2/Bax ratio notably increased in the HC, LNC, and HNC groups, indicating an improved anti-apoptotic capacity in the ileum under heat stress conditions (Figure 2D).

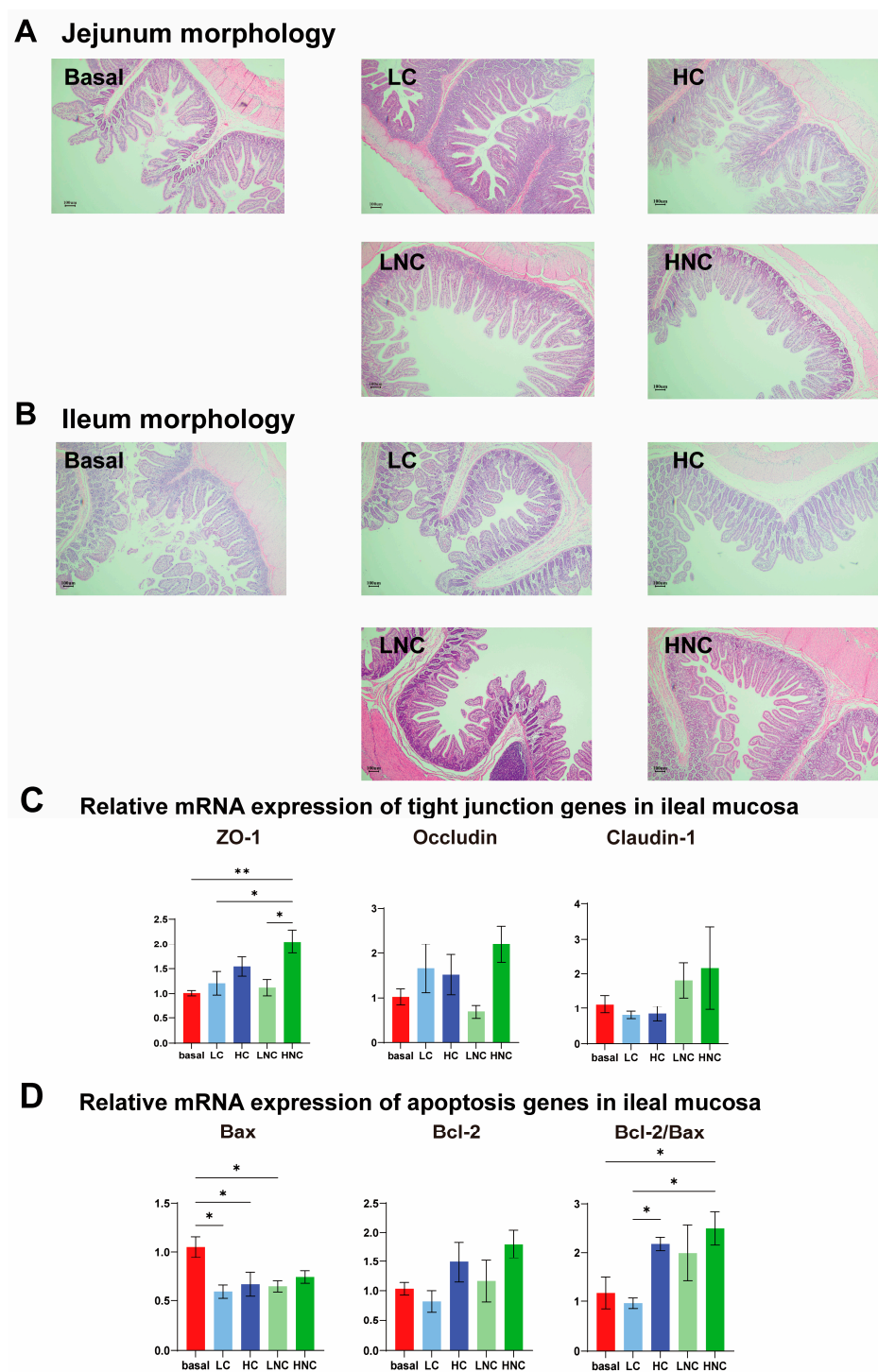


Figure 2. Effect of dietary Cu concentrations and forms on intestinal health of weanling pigs in heat stress. (A) Jejunum morphology; (B) Ileum morphology; (C) Relative mRNA expression of tight junction genes in ileal mucosa; (D) Relative mRNA expression of apoptosis genes in ileal mucosa. Data are presented as mean $\pm$ SEM, $n = 8$. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. * indicates significant difference ($p < 0.05$), ** indicates highly significant difference ($p < 0.01$), and Basal, basal diet with heat stress; LC, low dose of CuSO_4 , 50 mg/kg CuSO_4 in basal diet with heat stress; HC, high dose of CuSO_4 , 150 mg/kg CuSO_4 in basal diet with heat stress; LNC, low dose of nano- CuO , 50 mg/kg nano- CuO in basal diet with heat stress; HNC, high dose of nano- CuO , 150 mg/kg nano- CuO in basal diet with heat stress. ZO-1 = zonula occludens-1, Bax = B-cell lymphoma-2-associated X protein, Bcl-2 = B-cell lymphoma-2.

3.5. Anti-Oxidation

The results indicate that both copper sulfate and nano-copper supplementation enhanced the antioxidant capacity of the ileal mucosa in weanling pigs. Compared to the basal group, MDA concentrations in the jejunal mucosa were significantly lower in the HC, LNC, and HNC groups ($p < 0.05$, $p < 0.01$, Figure 3A), suggesting reduced lipid peroxidation. Additionally, both nano-copper treatments (LNC and HNC) significantly increased SOD activity in the mucosa compared to the control ($p < 0.05$), although no significant differences were observed in GSH-Px or T-AOC. Interestingly, GSH-Px activity was significantly decreased in the HC group ($p < 0.01$), indicating a potential adverse effect of high-dose copper sulfate on this antioxidant enzyme.

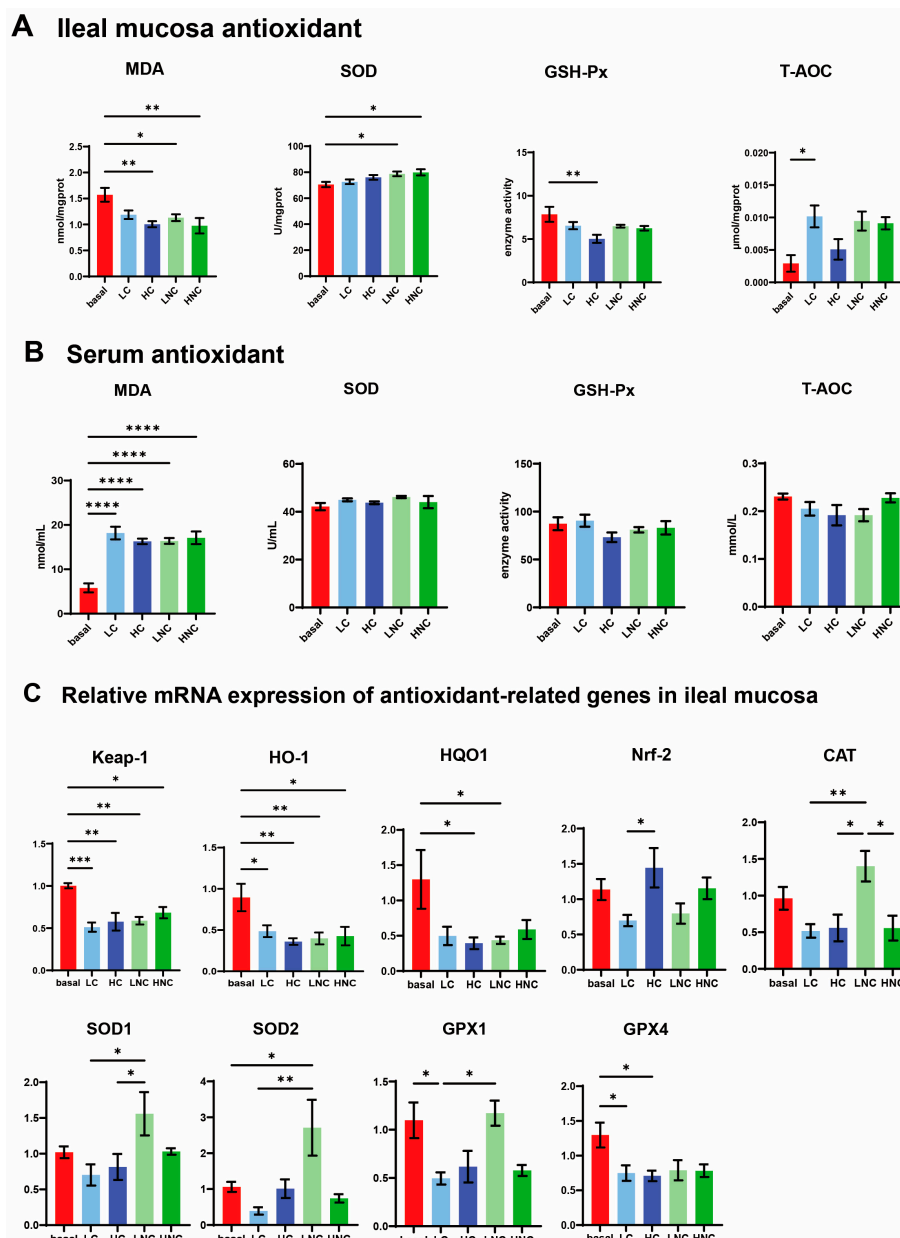


Figure 3. Effect of dietary Cu concentrations and forms on anti-oxidation of weanling pigs in heat stress. (A) Ileal mucosa antioxidant; (B) Serum antioxidant; (C) Relative mRNA expression of antioxidant-

related genes in ileal mucosa. Data are presented as mean $\pm$ SEM, $n = 8$. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. * indicates significant difference ($p < 0.05$), ** indicates highly significant difference ($p < 0.01$), *** indicates extremely significant difference ($p < 0.001$), and **** indicates $p < 0.0001$. Basal, basal diet with heat stress; LC, low dose of CuSO₄, 50 mg/kg CuSO₄ in basal diet with heat stress; HC, high dose of CuSO₄, 150 mg/kg CuSO₄ in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. MDA = malondialdehyde, SOD = superoxide dismutase, GSH-Px = glutathione peroxidase, T-AOC = total antioxidant capacity, Keap-1 = Kelch Like ECH Associated Protein 1, HO-1 = heme Oxygenase-1, NQO1 = NAD(P)H quinone oxidoreductase 1, NRF2 = nuclear respiratory factor 2, GPX4 = glutathione peroxidase 4, GPX1 = glutathione peroxidase 1, CAT = catalase, SOD1 = superoxide dismutase 1, SOD2 = superoxide dismutase 2.

In serum (Figure 3B), MDA levels were significantly higher in all copper-treated groups than in the control ($p < 0.001$), while no significant differences were found in SOD, GSH-Px, or T-AOC activities among groups.

At the gene expression level (Figure 3C), all copper-treated groups exhibited significantly lower expression of Keap-1, HO-1, and NQO1 ($p < 0.05$, $p < 0.01$, $p < 0.001$), suggesting an alleviation of oxidative stress in the ileal mucosa. Meanwhile, the mRNA expression of SOD1, SOD2, and CAT was significantly upregulated by nano-copper treatments, with 50 mg/kg of nano-copper (LNC) showing the highest SOD1 expression ($p < 0.05$). However, all copper treatments significantly reduced GPX4 expression ($p < 0.05$), which may indicate a potential antagonistic interaction between copper and glutathione peroxidase activity.

3.6. Inflammation

The results indicate that copper supplementation can modulate intestinal inflammation in weanling pigs under heat stress. Compared to the basal group, all copper-supplemented treatments significantly increased serum IFN- γ concentrations ($p < 0.001$, Figure 4A). Additionally, serum TNF- α was significantly elevated in the high-dose nano-copper group (HNC) compared to the other treatment groups ($p < 0.05$), suggesting that excessive copper nanoparticle supplementation may induce a pro-inflammatory response. In terms of heat stress alleviation, serum HSP70 levels were significantly reduced in both LC and HNC groups compared to the basal group ($p < 0.01$, Figure 4A), while mucosal HSP70 concentrations were also decreased in the LC group ($p < 0.01$, Figure 4B). These findings indicate that both low- and high-dose nano-copper may contribute to improved stress resilience. At the mRNA level in the ileal mucosa (Figure 4C), supplementation with both copper sulfate and nano-copper significantly downregulated the expression of pro-inflammatory cytokines IL-1 β and IL-6 ($p < 0.05$ or $p < 0.01$) compared to the basal group. Furthermore, IL-10 expression was significantly higher in the LC group compared to the HC, LNC, and HNC groups ($p < 0.01$), suggesting that low-dose nano-copper may exert a more pronounced anti-inflammatory effect than other treatments.

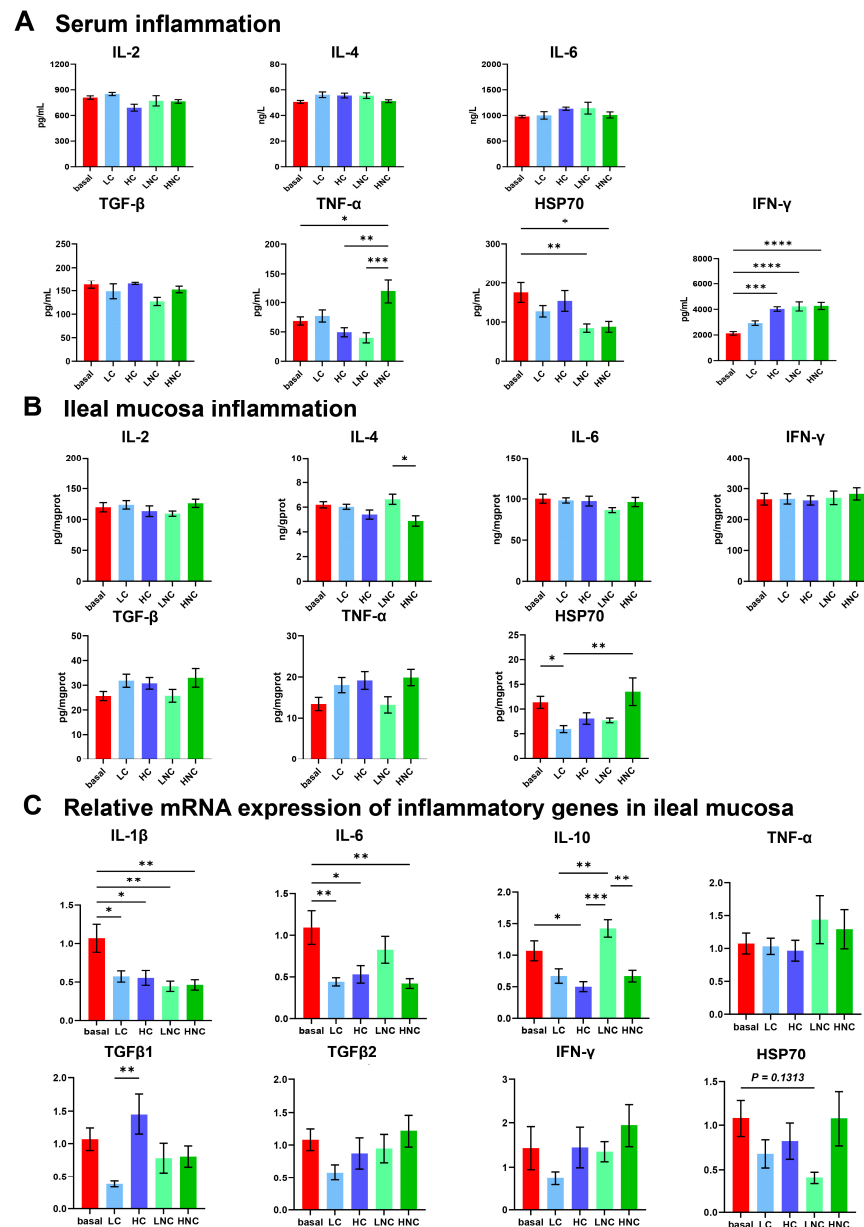


Figure 4. Effect of dietary Cu concentrations and forms on inflammatory cytokines of weanling pigs in heat stress. (A) Concentration of serum inflammatory cytokine; (B) Concentration of ileal mucosa inflammatory cytokine; (C) Relative mRNA expression of inflammatory genes in ileal mucosa. Data are presented as mean $\pm$ SEM, $n = 8$. Statistical analysis was performed using one-way analysis of variance (ANOVA), and intergroup differences were compared using Duncan's multiple range test. * indicates significant difference ($p < 0.05$), ** indicates highly significant difference ($p < 0.01$), *** indicates extremely significant difference ($p < 0.001$), and **** indicates $p < 0.0001$. Basal, basal diet with heat stress; LC, low dose of CuSO_4 , 50 mg/kg CuSO_4 in basal diet with heat stress; HC, high dose of CuSO_4 , 150 mg/kg CuSO_4 in basal diet with heat stress; LNC, low dose of nano-CuO, 50 mg/kg nano-CuO in basal diet with heat stress; HNC, high dose of nano-CuO, 150 mg/kg nano-CuO in basal diet with heat stress. TGF- β = transforming growth factor- β , TNF- α = tumor necrosis factor α , IL-1 β = interleukin-1 β , IL-2 = interleukin-2, IL-4 = interleukin-4, IL-6 = interleukin-6, IL-10 = interleukin-10, IFN- γ = interferon γ , HSP70 = heat shock protein 70, TGF- β 1 = transforming growth factor- β 1, TGF- β 2 = transforming growth factor- β 2.

4. Discussion

Excessive use of high-concentration copper (Cu) in livestock feed and fertilizers has led to growing environmental concerns, particularly due to copper accumulation in manure.

Studies have shown that pig manure is a major source of copper input in agricultural soils, especially in intensive production regions like Beijing and Fuxin, where elevated soil copper levels pose a risk of farmland pollution [11]. Beyond soil contamination, copper excretion affects microbial communities and may promote the emergence of copper-resistant bacteria, potentially contributing to the spread of antibiotic resistance genes [6,8,21]. Climate issues further complicate the situation, especially in regions like Southwest China, where the summer temperature and humidity are particularly high. High ambient temperatures induce heat stress in livestock, which can impair their physiological functions, including the absorption of essential mineral elements such as copper [10]. Heat stress is known to compromise the immune function and intestinal integrity of animals, reducing their ability to effectively utilize dietary minerals [22]. This results in increased copper excretion and further environmental contamination. After the ban on antibiotics, copper has been considered an effective alternative additive. Dietary Cu can stimulate piglet growth and alter the intestinal microbiota by its antimicrobial effect [4,23]. Our study shows that nano-copper supplementation, both at low (50 mg/kg) and high (150 mg/kg) doses, significantly improves the growth performance of weaned piglets under heat stress conditions. These findings align with previous research demonstrating that copper enhances weight gain and feed efficiency in pigs.

The absorption rate of copper in animals is inversely proportional to the intake level. When copper intake is very high, the absorption rate can be as low as 12%. In humans, the ability to absorb copper increases with age. In suckling rats, more copper is retained in the intestinal mucosa after copper infusion compared to adult rats [24]. Piglets have higher fecal copper content compared to finishing pigs and sows [21]. So, the excessive addition of copper in feed is not appropriate. In former studies, different forms of copper were added to the feed in doses of 125 to 250 mg/kg [4,25,26]. Our study indicates that two different copper supplementations, both at low (50 mg/kg) and high (150 mg/kg) doses, significantly improve the growth performance of weaned piglets under heat stress conditions (Table 3). These findings align with previous research demonstrating that copper enhances weight gain and feed efficiency in pigs [27]. The enhanced nutrient digestibility observed in piglets fed with nano-copper is noteworthy. This improvement can be attributed to the role of copper in enzymatic functions and its impact on the gut microbiota. Copper's antimicrobial properties help maintain a healthier gut environment, promoting better nutrient absorption [28]. Notably, the low-dose nano-copper group exhibited the highest digestibility rates (Table 4), indicating that lower concentrations of nano-copper can achieve optimal results without the need for higher dosages, thus reducing the risk of copper accumulation in the environment. The measurement of copper content in feed and feces reveals a significant advantage of nano-copper over copper sulfate in terms of bioavailability (Table 5). The lower fecal copper content in the nano-copper groups suggests that nano-copper is more efficiently utilized by the piglets, reducing the excretion of unmetabolized copper into the environment. This finding indicates that nano-copper supplementation can lower the environmental burden of copper without compromising animal health and growth performance.

Nano-copper's influence on intestinal health and antioxidant capacity is particularly significant under heat stress conditions, which are known to compromise intestinal integrity by increasing permeability and inducing inflammation [29,30]. Our findings demonstrate that nano-copper, particularly at low doses, significantly enhances the antioxidant capacity of intestinal tissues, thereby protecting against oxidative damage caused by heat stress. This protective effect is crucial for maintaining gut health and the overall well-being of the animals. The improved intestinal morphology observed in the low nano-copper group, with increased villus height and reduced crypt depth, further supports the beneficial im-

pect of nano-copper on gut health (Figure 2A, Table 6). Enhanced villus height improves nutrient absorption, while reduced crypt depth indicates lower rates of cellular turnover and inflammation. This aligns with previous studies indicating that copper plays a role in maintaining intestinal integrity and function [25,31]. Moreover, our data suggest that low-dose nano-copper exhibits anti-inflammatory properties, as evidenced by the reduced expression of inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α (Figure 4C). In contrast, high-dose nano-copper induces a pro-inflammatory response, likely due to excessive accumulation of copper, which can lead to oxidative stress and activation of inflammatory pathways (Figure 4A,B). This dual effect highlights the necessity of optimizing copper dosage to harness its benefits while avoiding adverse effects. Studies have shown that excessive copper can disrupt the balance of pro- and anti-inflammatory cytokines, leading to an inflammatory response [17]. The antioxidant capacity is further supported by the elevated expression of genes associated with antioxidative functions, such as SOD1 and GPX1, in the low nano-copper group (Figure 4C). These genes are crucial for detoxifying reactive oxygen species and protecting intestinal tissues from oxidative damage. Conversely, high-dose nano-copper may overwhelm the antioxidative defense system, resulting in oxidative stress and inflammation (Figure 4A).

Nano-copper may exert its beneficial effects through the coordinated modulation of redox balance, immune response, and epithelial integrity. Under heat stress conditions, excessive reactive oxygen species (ROS) impair epithelial function and trigger inflammatory signaling cascades [32–34]. The observed increase in antioxidant enzymes (SOD, CAT) and decrease in MDA suggests that nano-copper enhances endogenous antioxidant defenses, likely through improved copper-dependent enzyme activity and reduced oxidative burden [35–37]. This redox improvement may directly contribute to the preservation of tight junction proteins (e.g., ZO-1 and Occludin), thereby maintaining intestinal barrier integrity [38,39]. Simultaneously, the downregulation of pro-inflammatory cytokines (e.g., IL-1 β , TNF- α) may be a consequence of reduced oxidative stress and restored epithelial function, since ROS are known to activate NF- κ B-mediated inflammatory pathways [40]. Furthermore, improved gut morphology (increased villus height and villus-to-crypt ratio) indicates enhanced absorptive capacity, which may be sustained by the anti-inflammatory and antioxidant environment in the intestinal mucosa. Therefore, nano-copper appears to act via a synergistic mechanism: reducing oxidative stress, suppressing inflammation, stabilizing barrier function, and ultimately improving nutrient absorption and growth performance. These findings suggest that nano-copper not only improves copper utilization efficiency but also enhances systemic resilience under heat stress through multiple interconnected physiological pathways. This mechanistic understanding strengthens the case for using nano-copper as a functional additive in swine nutrition under challenging environmental conditions.

These findings have potential implications for commercial swine production, especially in regions frequently exposed to high ambient temperatures. In real-world settings, low-dose nano-copper supplementation may serve as a practical strategy to mitigate heat stress-induced performance losses while reducing copper excretion into the environment. This dual benefit could support more sustainable pig farming practices in line with current environmental regulations and animal welfare standards.

However, our findings must be interpreted within the study's limitations. The 22-day trial duration precludes assessment of long-term nanoparticle accumulation in organs, necessitating chronic toxicity studies. Although we measured copper concentrations in serum and feces, we did not evaluate hepatic copper levels. Given the liver's central role in copper metabolism and storage, future studies should assess tissue distribution to better understand the bioaccumulation profile of nano-copper. Additionally, the controlled

environment (constant 35 °C) may underestimate real-world variability in humidity and diurnal temperature fluctuations. Future research should integrate multi-omics approaches to unravel nano-CuO's interaction with gut microbiota and host metabolism, particularly under cyclic heat stress conditions.

5. Conclusions

Nano-copper exhibits the same promoting effect on piglet growth, diarrhea alleviation, and increased nutrient digestibility as traditional copper sulfate. It can also enhance the antioxidant and anti-inflammatory abilities of weanling pigs' intestines, alleviate heat stress, and improve gut health. Notably, low-dose nano-copper has a better effect on enhancing antioxidant capacity, while high-dose nano-copper may have some pro-inflammatory effects. The determination of copper content in feed and feces shows that 50 mg/kg of nano-copper can improve weanling pigs' copper utilization while reducing fecal copper content. In conclusion, low-dose nano-copper appears to be a promising alternative to traditional copper sulfate, as it improves piglet growth performance and antioxidant status while reducing fecal copper excretion.

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Institutional Review Board Statement: The animal study protocol was approved by Sichuan Agricultural University Animal Ethical and Welfare Committee. (Approval No. 20210102).

Data Availability Statement: The data will be made available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ADFI	Average daily feed intake
ADG	Average daily gain
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATTD	Apparent total tract digestibility
BAD	Bcl-2-associated death promoter
Bax	B-cell lymphoma-2-associated X protein
Bcl-2	B-cell lymphoma-2
BUN	Blood urea nitrogen
Ca	Calcium
Caspase-3	Cysteine-aspartic acid protease-3
Caspase-8	Cysteine-aspartic acid protease-8

Caspase-9	Cysteine-aspartic acid protease-9
CAT	Catalase
CP	Crude protein
DM	Dry matter
EE	Ether extract
F:G	Feed-to-gain ratio
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPX1	Glutathione peroxidase 1
GPX4	Glutathione peroxidase 4
GSH-Px	Glutathione peroxidase
HC	High copper sulfate
HNC	High nano-copper
HO-1	Heme oxygenase-1
HSP70	Heat shock protein 70
IFN- γ	Interferon- γ
IL-10	Interleukin-10
IL-1 β	Interleukin-1 β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
Keap-1	Kelch-like ECH-associated protein 1
LC	Low copper sulfate
LNC	Low nano-copper
MDA	Malondialdehyde
NQO1	NAD(P)H quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
STTD	Standard total tract digestibility
T-AOC	Total antioxidant capacity
TGF- β	Transforming growth factor- β
TNF- α	Tumor necrosis factor- α
TP	Total phosphorus
T-SOD	Total superoxide dismutase
ZO-1	Zonula occludens-1

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