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The Application of Copper Waterline on Laying Performance and Gut Health of Aged Laying Hens

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The effect of the application of copper waterline on the performance and gut health of aged laying hens was evaluated in this study. Forty-eight 70-week-old laying hens were divided into two groups (three replicates of eight hens each): control and copper (Cu) groups provided with normal polyvinyl chloride (PVC) waterline or Cu waterline. The laying performance was measured during the four-week period of the experiment. The intestinal antioxidant status and the microbiota diversity of the cecal content were determined. Moreover, a bacteriostasis test on *Escherichia coli* and *Salmonella enteritidis* was conducted after inoculation in waterline and hens, respectively. The water Cu^{2+} content was increased by Cu waterline compared to the control ($P<0.05$). Cu waterline had no detectable effect on most production performances, however, it increased the egg weight ($P<0.05$). Cu waterline increased the Cu level in the eggshell. Cu level in excreta increased with time, especially in the final two weeks, however, there was no significant change in fecal Cu excretion. The lipid peroxidation product malondialdehyde content in ileum decreased ($P<0.01$), while the activities of CuZn-superoxide dismutase (SOD) of ileum and glutathione peroxidase (GSH-PX) activity of jejunum and ileum increased after Cu treatment. The relative abundance and richness of cecal microbiota increased after Cu treatment ($P<0.05$). Cu waterline changed the microbial composition, including the increased proportion of *Methanocorpusculum*, *Paludibacter*, and decreased proportion of *Fucobacterium*, *Anaerobiospirillum*, *and Campylobacter*. The colonization of *E. coli* and *S. enteritidis* in Cu waterline was suppressed by Cu treatment, indicating that Cu waterline had potential antibacterial properties. The result suggests that Cu waterline could inhibit the colonization of pathogenic microorganisms such as *E. coli* and *Salmonella* and facilitate the enrichment of cecal microbiota diversity.

Key words: copper waterline, antioxidant capacity, microbial diversity, antibacterial activity, laying hens

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Introduction

Copper (Cu) is essential in several enzyme systems, including cytochrome oxidase, tyrosinase, lysyl oxidase, and superoxide dismutase, by serving as co-factor of a variety of intracellular and extracellular enzymes (Klasing 1998). The Cu requirement for laying hens is not available in the National Research Council (1994) list. Dietary Cu supplementation level had no significant influence on the laying performance of hens but decreased serum cholesterol concentration, suggesting that 125 mg/kg of Cu provide adequate supplementary concentrations for laying hens (Balevi and Coskun 2004; Lien *et al*., 2004). For late-phase hens, a corn-soybean basal diet (containing 10.3 mg Cu/kg) might be sufficient to meet their maintenance and production requirements (Li *et al*., 2018). The lack of copper in laying hens leads to anemia and abnormal egg size and shape (Baumgartner *et al*., 1978).

In practice, Cu addition at prophylactic levels is used as a growth promoter in poultry production (Leeson, 2009; Bortoluzzi *et al*., 2020). However, high levels of Cu supplementation may have two negative effects: oxidative damage induced by Cu overloading (Zhang *et al*., 2000; Toplan *et al*., 2005) and elevated excretion of Cu in the excreta (Skrivan *et*

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al., 2006). More than 90% of copper is excreted with droppings when supplemented at a pharmacological concentration (Mohanna and Nys, 1998), leading to heavy metal waste in livestock and poultry breeding.

The bactericidal action of Cu depends on the concentration of free ionic Cu in solution (Zevenhuizen *et al*., 1979). Acidic copper sulfate-based commercial sanitizer is successfully used at various intervention points of poultry processing (Russell, 2008). It has recently been proven that Cu could reduce the conjugative transfer of resistance plasmids from extended-spectrum β-lactamase-producing *E. coli* (Buberg *et al*., 2020). *In vivo*, supplementation with 187.5 mg/kg of Cu from Cu sulfate pentahydrate had no effect on the number of ileal lactobacilli of birds (Pang *et al*., 2009).

In this study, the use of a copper waterline was evaluated in laying hens. The laying performance, Cu intake and excretion, antioxidant capacity, and microbial composition in cecal content were measured. The inhibition of *E. coli* and *S. enteritidis* by Cu waterline was respectively measured *in vitro* and *in vivo*. *E. coli*, a pathogenic bacteria that exists widely in animals and nature, was used to detect the inhibitory effect of Cu waterline on the spread of bacteria. *S. enteritidis*, an epidemic pathogen, was employed to evaluate the inhibitory effect of Cu waterline on the bacterial colonization in the gut of chickens. Our research sheds new light on copper waterline applications in aged laying hens by explaining its potential effect of improving antioxidative and antibacterial effects and changes in microbial composition.

Materials and Methods

Animal Ethics

All study procedures were approved by the Animal Care and Use Committee of Shandong Agricultural University (SDAUA-2022-65) and were in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, P. R. China).

Birds and Experiment Design

Forty-eight 70-weeks old Hy-line Brown laying hens with an average body weight of 2.25±0.01 kg were randomly assigned to two groups and reared in cages equipped with a normal poly vinyl chloride (PVC) waterline (control) or copper waterline (Cu). Each treatment had three replicates of eight hens. The laying hens were reared in cages (60-cm length×45-cm width×50-cm height), with two birds in each cage. Drinking water was provided using nipple drinkers (1 nipple/cage.) via waterline. Duplicate water samples (10 mL) were obtained from each nipple drinker 4 times (6 repeats each time) every week from the control and Cu waterlines, and the water samples were mixed and stored at 4℃ for further measurement. All laying hens had free access to feed and water. The lighting program was 16L:8D. The experimental diet (Table 1) was formulated to meet the recommendation by NRC (1994). The experiment lasted four weeks. Feed intake, water intake, and egg production were recorded daily. The feces were collected daily for every replicate. After drying and weighing, the Cu content was measured, and the average daily fecal Cu excretion was calculated. At the end of the experiment, blood samples were obtained from the wing vein using heparinized syringes and placed into ice-cold tubes. Plasma was obtained after centrifugation at 400 *g* for 10 min at 4℃ and was stored at −20℃ for further analysis. After the blood samples were obtained, laying hens were killed by exsanguination (Close, 1997; Wang *et al*., 2020). The content of the cecum was collected, snap-frozen in liquid nitrogen, and stored at −80℃. The duodenum, jejunum, and ileum mucosae were collected, snap-frozen in liquid nitrogen, and stored at −80℃ for enzyme activity assay.

Table 1. **Ingredient and nutrition composition of the basal diet**

Ingredient	Content %
Corn	59.65
Soybean meal	22.91
Wheat bran	5.00
Soya-bean oil	1.14
Limestone powder, ground	8.93
Calcium hydrophosphate	1.53
Iodized salt	0.35
Lysine (99%)	0.04
Methionine (98%)	0.10
Choline chloride (50%)	0.10
Vitamin mix[1]	0.05
Mineral mix[2]	0.20
Nutrition level[3]	
ME, kcal/kg	2650
Crude protein, %	16.5
Calcium, %	3.54
Available phosphorus, %	0.40
Crude fiber, %	2.6
Cu mg/kg[4]	27.16

[1] Vitamin premix provided (per kilogram of diet): Vitamin A (from retinyl acetate), 5,000 IU; vitamin B1, 345 mg; vitamin B2, 1,375 mg; Vitamin D3, 1000 IU; Vitamin E, 6,600 mg; Vitamin K, 458 mg; Pantothenic acid, 1,340 mg; Nicotinic acid, 5,658 mg; Pyridoxine, 673 mg; Biotin, 550 mg; Folic acid, 122 mg; Cobalamin, 442 mg.
[2] Mineral premix provided (per kilogram of diet): $MnSO_4$, 13.84 g; $ZnSO_4$, 12.75 g; $FeSO_4$, 9.17 g; Na, 33.56 mg; KI, 113.48 mg.
[3] Calculated values.
[4] Measured value.

Microbial Analysis of the Cecal Contents

Total bacterial genomic DNA samples were extracted, and the quantity and quality were measured. After the detection of DNA, the PCR amplification of the bacterial 16S rRNA gene V4-V5 region was performed on the IIIumina Miseq PE250 platform. After initial denaturation, annealing, and extension, the PCR amplicons were obtained, purified, quantified, and pooled in equal amounts. The separation and amplification 16S rDNA and quantification of the microflora composition were performed at the Annoroad Gene Technology Company (Beijing, China). The average number of observed operational taxonomic units (OTU) at 97% identity was determined using UCLUST in QIIME software (Version 1.9.1). To estimate the portion of the diversity covered by our subsampling, the diversity of the OTUs found in the samples and their evenness were measured. The OTUs were taxonomically annotated based on the Silva (http://www.arbsilva.de) taxonomic databases. The QIIME software was used to generate the species abundance table at different levels (phylum, class, order, family, genus, species) and displayed with R software (Version 2.15.3). The alpha diversity of samples (Chao 1, Shannon index, Rank abundance curve) was analyzed using R software. To assess beta diversity, the unweighted UniFrac distance metrics were produced by QIIME software, and principal coordinate analysis (PCoA) was performed.

Cu Analysis

Diet, fecal, and serum Cu levels were analyzed by graphite furnace atomic absorption as described by Meeravali and Arunachalam (1997). Cu concentration in water was determined by ICP-AES 7000SERIES atomic absorption spectrometer (Thermo Fisher) at an absorption wavelength of 324.7 nm.

T-AOC, CuZn-SOD, GSH-PX and Malondialdehyde (MDA) Measurement

The activity of total superoxide dismutase (T-SOD), CuZn-SOD, total antioxidant activity (T-AOC), and activity of glutathione peroxidase (GSH-PX) of the serum and intestinal mucosa homogenate were measured with commercial kits (Jiancheng Bioengineering Institute, Nanjing). The level of lipid peroxides released from the small intestinal tracts, estimated as the level of lipid peroxides released from the small intestinal tracts, was estimated as TBARS according to Huang *et al*. (2015).

Escherichia coli Bacteriostasis Test

E. coli was obtained from the Avian Disease Centre of Shandong Agricultural University (Shandong, China). The *E. coli* strain was seeded on LB (Solarbio, China) agar to obtain isolated pure colonies, and a single colony was inoculated into LB broth and incubated overnight at 37℃ while shaking at 180 *rpm* (QYC-2102, Fuma, China) for 10 h. The concentration of *E. coli* was adjusted to 8.22×10^6 colony-forming unit (CFU)/mL in sterile saline.

Three pipes of Cu waterline and control waterline (1.8 m long) filled with tap water were respectively injected with 1 mL sterile saline or *E. coli* solution at a concentration of 8.22×10^6 CFU/mL. Afterward, a water sample was obtained every 24 h for five times. The water samples (100 µL) were serially diluted 10-fold with sterile phosphate-buffered saline (1:10, w/v), and then screened on MacConkey plates (Hopebio, Qingdao, China). After incubation at 37℃ for 24 h, the CFU of *E. coli* was enumerated.

Salmonella Challenge Test

A strain of *S. enteritidis* (SDJX-1) isolated from chickens was obtained from the Avian Disease Centre of Shandong Agricultural University. A single colony was selected from the xylose lysine deoxycholate agar plate and transferred into a tube containing 5 mL tryptic soy broth and then incubated at 37℃ while shaking at 180 rpm (QYC-2102, Fuma, China) for 16 h. The concentration of *S. enteritidis* was adjusted to 2.53×10^5 CFU/mL in sterile saline.

Ten one-day-old SPF White Leghorn layer-type chicks were obtained from Jinan SPAFAS Poultry Company (Jinan, China). The chickens were reared individually in a cage within an environmentally controlled chamber. Each cage had a feeder and nipple drinker. The chicks were fed a commercial diet and had free access to feed and water throughout the experimental period. On Day 8, the chicks were orally challenged with 0.2 mL of *S. enteritidis* (2.53×10^5 CFU/mL). Afterward, the chicks were randomly divided into two groups and were reared in cages equipped with a control waterline or Cu waterline. The experiment lasted 12 days, and a water sample was obtained every day from every nipple ($n=5$ for each treatment). The water sample (100 µL) was serially diluted 10-fold with sterile phosphate-buffered saline (1:10, w/v), screened on xylose lysine deoxycholate plates (Hopebio, Qingdao, China), incubated at 37℃ for 24 h, and then the CFU of *S. enteritidis* was enumerated.

Statistical Analysis

Prior to analysis, all data were examined for the homogeneity and normal distribution plots of variances among the treatments by using the UNIVARIATE procedure. The main effect was evaluated using a one-way ANOVA with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC, USA). The antibacterial effect against *Salmonella* was analyzed by two-way ANOVA with SAS software. The data of 16S rRNA sequencing were calculated with QIIME (Version 1.9.1) and displayed with R software (Version 2.15.3). $P<0.05$ was considered statistically significant.

Results

There were no significant differences in body weight, average feed intake, water intake, and egg production between the control and Cu groups. Egg weight, however, increased with Cu treatment ($P<0.05$, Table 2). Cu treatment increased the Cu level in the water and Cu intake from water ($P<0.01$, Table 3). Similarly, the daily Cu intake significantly increased ($P<0.01$). Cu waterline had no significant influence on Cu concentration in serum and egg content, whereas increased Cu level was observed in the eggshell ($P<0.05$, Table 3). Cu level in the excreta was not significantly changed by the Cu treatment in the first and second week but increased at Week 3 ($P<0.05$) and Week 4 ($P<0.1$, Fig. 1A). In contrast, the average daily fecal Cu excretion during the four-week period was not significantly changed by Cu treatment (Fig. 1B).

Cu treatment had no significant influence on serum T-SOD and CuZn-SOD activity and T-AOC (Fig. 2A, B, C). The activity of CuZn-SOD did not change in the duodenum and jejunum but increased ($P<0.05$) with Cu treatment in the ileum (Fig. 3 A). In contrast, the activities of T-SOD in the duodenum, jejunum, and ileum mucosa did not change with Cu treatment (Fig. 3B), same as the T-AOC level ($P=0.139$, 0.311, and 0.820, Fig. 3C). GSH-PX activity increased with Cu treatment in the jejunum and ileum ($P<0.05$) but not in the duodenum (Fig. 3D). MDA level was not influenced in the duodenum and jejunum, however, it decreased ($P<0.01$) with Cu treatment in the ileum (Fig. 3E). The protein carbonyl

Table 2. **Effect of copper waterline on the laying performance of hens**

	Control	Cu	P-Value
Egg production, %	74.4±2.15	75.6±2.59	0.741
Egg weight, g	66.0±0.37[b]	68.3±0.67[a]	0.039
Feed intake, g/hen/day	113.2±0.4	107.5±2.7	0.103
Feed efficiency	2.30±0.03[b]	2.08±0.04[a]	0.003
Water intake, mL/hen/day	278.3±48.3	313.7±42.1	0.610
Body weight, g	2.34±0.04	2.32±0.04	0.829

Data were presented as Mean±SEM (n=3);
[a, b] Means within the same line with different superscript differ significantly (P<0.05).

Table 3. **Effect of copper waterline on Cu intake and Cu content in serum and egg**

	Control	Cu	P-Value
Cu intake			
Cu content in diet, mg/kg	27.16	27.16	
Dietary Cu intake, mg/hen/d	3.07±0.01	2.92±0.07	0.103
Water Cu content, mg/L	0.043±0.02[b]	0.373±0.06[a]	0.008
Cu intake from water, mg/hen/d	0.012±0.002[b]	0.117±0.016[a]	0.003
Total Cu intake, mg/d	3.09±0.01	3.04±0.06	0.536
Serum Cu level, µg/mL	1.16±0.06	1.41±0.16	0.178
Cu level in egg content, µg/g	2.32±0.76	4.23±0.14	0.235
Cu level in eggshell, µg/g	7.13±0.26[b]	8.08±0.27[a]	0.019

Values are presented as Mean±SEM (n=6).
[a, b] Means within the same line with different superscripts differ significantly (P<0.05).

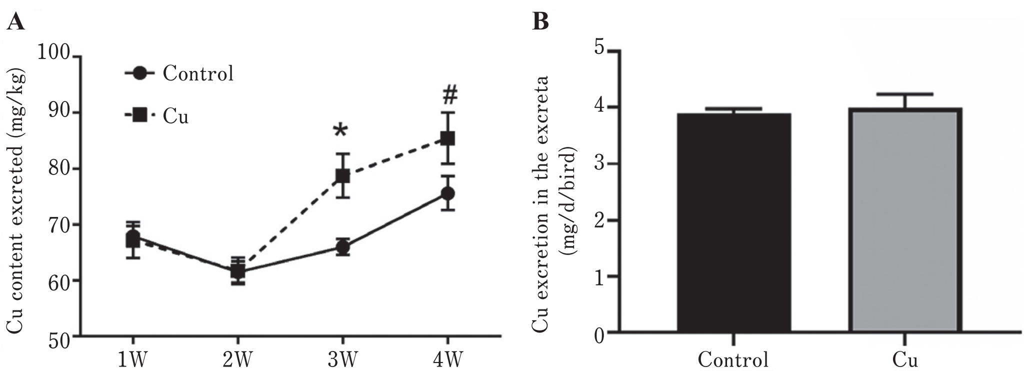

Fig. 1. **Effect of copper waterline on copper content in excreta of laying hens.** (A) fecal Cu concentration (mg/kg), and (B) average daily fecal Cu excretion (mg/d/bird). Data are shown as Mean±SEM (n=3); * P<0.05, # 0.05<P<0.1.

concentration was not altered by Cu treatment (Fig. 3F).

Alpha diversity was applied to analyze the complexity of species diversity per sample. Chao 1 diversity analysis showed that Cu treatment increased (P<0.05) the alpha diversity of cecal microbiota (Fig. 4A). The Shannon index in Cu-hens showed an increasing tendency (Fig. 4B). Furthermore, the rank abundance curve was longer on the X-axis for Cu-administered laying hens, thus, verifying the increased microbiota diversity in Cu-hens (Fig. 4C). Consistent with the alpha diversity, the microbial composition increased significantly at order, family, and species levels in Cu-hens, compared with the control (P<0.05). The Cu-hens had 36, 53, and 17 more genera at order, family, and species levels than the control hens (P<0.05, Fig. 4D). The cecal microbiota was characterized by 18 major phyla, in which *Firmicutes* and *Bacteroides* were the most predominant phyla. Although there was no significant difference in the abundance of major phyla between the Cu-treated and control groups, in Cu-hens, the respective abundance of phylum *Elusimicrobia* increased and phylum *Fusobacteria* decreased (0.12% and 4.63%) compared with the control (0.03% and 8.84%, Fig. 4E). At the lower taxonomic levels, the genera, *Methanocorpusculum, Paludibacter, Elusimicrobium, Gracilibacter,* and *Syntrophomonas* (0.05%, 0.60%, 0.06%, 0.0008%, and 0.001%),

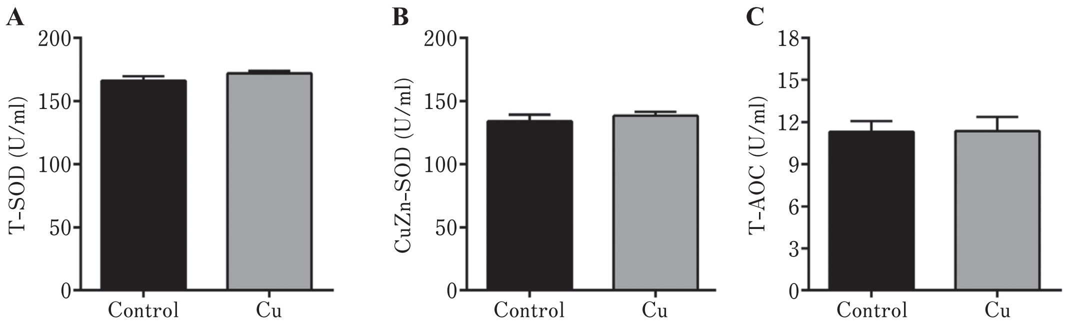

Fig. 2. **Effect of copper waterline on serum anti-oxidant properties.** (A) T-SOD, (B) CuZn-SOD, and (C) T-AOC activities. Values are presented as Mean±SD (n=8).

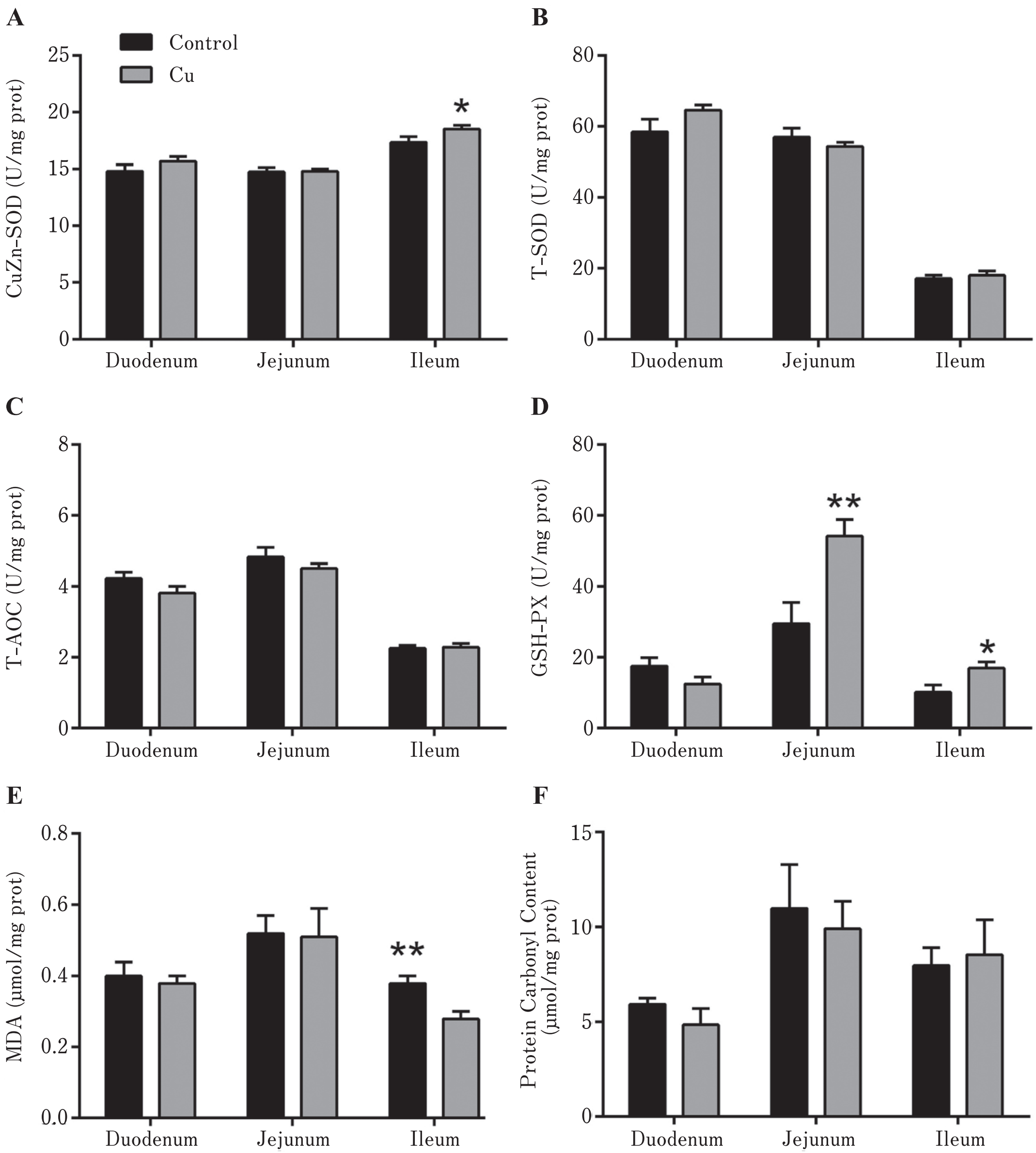

Fig. 3. **Effect of copper waterline on redox balance in duodenum, jejunum, and ileum mucosae.** (A) CuZn-SOD, (B) T-SOD, (C) T-AOC, (D) GSH-PX, (E) Content of MDA, and (F) Content of Protein Carbonyl. Data are presented as Mean±SD (n=8); * $P<0.05$, ** $0.05<P<0.01$.

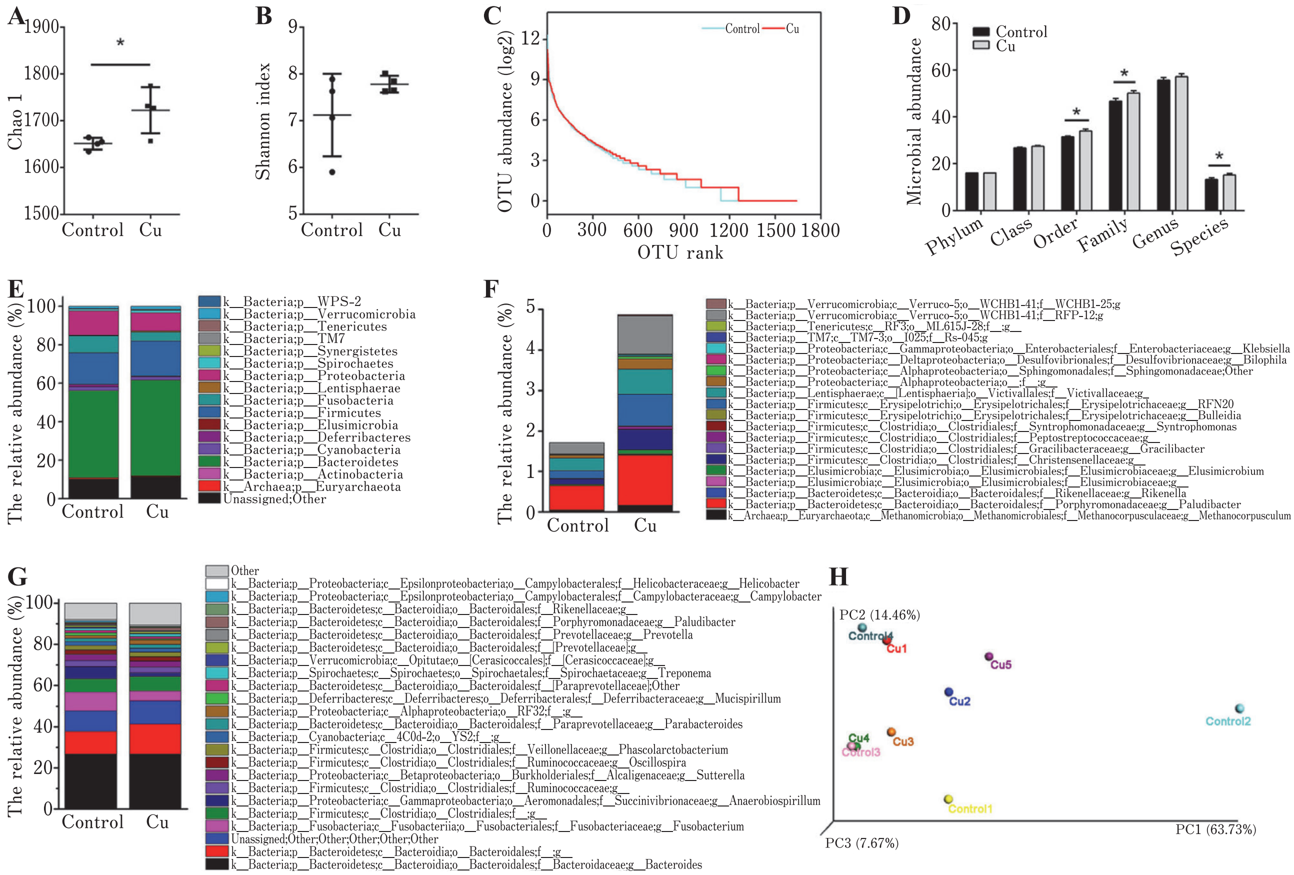

Fig. 4. **Effect of copper waterline on the alpha diversity and microbial abundance of microbiota in the cecal content of laying hens.** (A) Chao 1, (B) Shannon index, (C) Rank abundance curve, (D) OTU, (E) Phylum-level relative abundance (%) represented as a stacked graph, (F) The top 20 changes in genus-level relative abundance (%), (G) genus-level relative abundance (>0.5%) represented as a stacked graph, and (H) PCoA analysis based on unweighted UniFrac distance metric matrices for each individual hens among groups. Data are shown as Mean±SD ($n=4$); * $P<0.05$; ** $0.05<P<0.1$.

and the family, Peptostreptococcaceae (0.003%) were enriched in the cecal content of Cu-hens (0.17%, 1.24%, 0.11%, 0.0021%, 0.004% and 0.076%, Fig. 4F). Cu treatment respectively increased *Bacteroidales, Clostridiales,* and *Alphaproteobacteria* (14.61%, 6.90%, and 2.30%) and decreased *Fucobacterium, Anaerobiospirillum*, and *Campylobacter* (4.62%, 1.86%, and 0.13%) at different levels of relative abundance (over 0.5%) (Fig. 4G), compared with those in control group (11.07%, 6.38%, 2.10%, 8.84%, 6.03%, and 0.49%). The samples in the control group did not form a distinct cluster, but they were essentially separated from those in the Cu treatment in the PCoA plot (Fig. 4H), indicating that Cu treatment had a potential effect on cecal microbiota structure.

At 24 h time point after inoculation treatment, the *E. coli* content was significantly higher ($P<0.001$) in the inoculation groups, and there was no difference between the Cu and normal waterline groups (Fig. 5A). At 48 and 72 h after *E. coli* treatment, the *E. coli* content significantly decreased with Cu treatment ($P<0.001$) compared to the control (Fig. 5A). In the Cu treatment, the *S. enteritidis* content was significantly lower ($P<0.01$) than that of the control 3 d after inoculation (Fig. 5B).

Discussion

Copper is an essential mineral element for chickens. In broilers, copper promotes growth performance. Recently, based on meta-analysis, it was concluded that higher copper content is beneficial for the production performance of broilers (Feng *et al*., 2020).

In laying hen, copper deficiency results in anemia and eggs with abnormal sizes, shapes, and eggshells (Baumgartner *et al*., 1978). The hen-day egg production, egg weight, daily feed intake, and feed efficiency values were not affected by dietary levels of copper sulfate from 0 to 143 mg/kg in laying hens fed a corn-soybean basal diet that provided 6 mg Cu/kg diet (Christmasr and Harms, 1983). In contrast, supplementing hens with high Cu levels exceeding 250 ppm impairs the laying performance of hens by reducing feed consumption and decreasing the circulation of 17 β-oestradiol concentrations (Pearce *et al*., 1983).

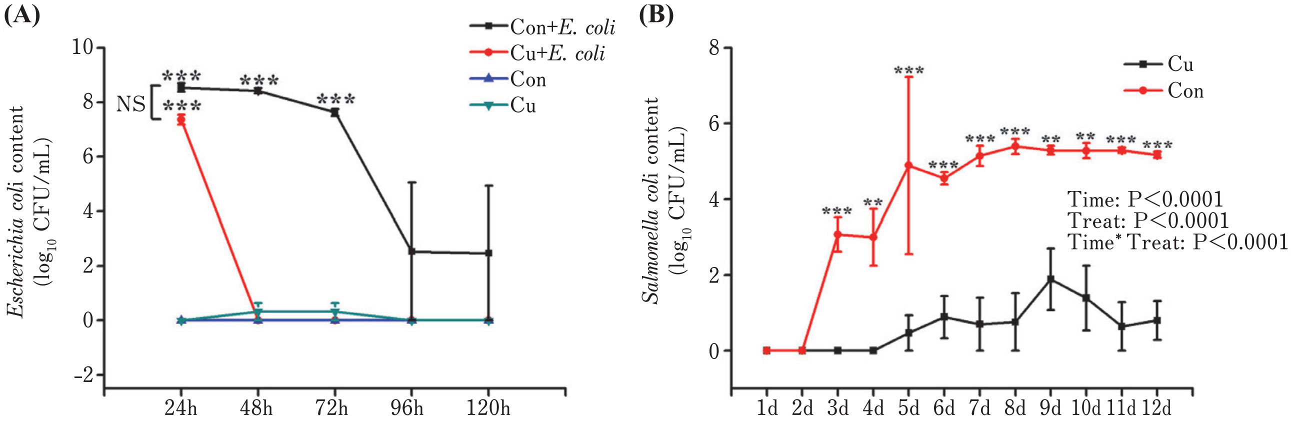

Fig. 5. **The antibacterial effect of copper waterline on (A) *E. coli* (*n*=3), and (B) *S. enteritidis* (*n*=5).** Data are shown as Mean ± SD; ** $0.05<P<0.01$; *** $P<0.0001$.

In this study, the laying rate and feed intake were not significantly altered while Cu treatment increased egg weight and feed efficiency (Table 2), indicating that the Cu waterline is beneficial for the laying performance of hens. This result was contrary to the previous result of high dose of dietary Cu by al Ankari *et al*. (1998), who reported that a significant reduction in egg production and a negative effect on food conversion was found when 250 mg/kg of copper was added compared to the control (no added copper). In the Cu treatment group, feed Cu intake was similar to that of the control hens, whereas Cu water intake was significantly higher than in the control hens (Table 3). The result implies that Cu source from water improves feed efficiency. Indeed, the quadratic response of food intake and the adverse effects on food intake, egg production, and body-weight has been observed following the addition of $CuSO_4$ but not the addition of CuO (Jackson and Stevenson, 1981).

The Cu levels in serum and egg content were not changed in the hens of the Cu group. This result was contrary to that observed in broiler breeder hens by Berwanger *et al*. (2018), who reported that serum and yolk Cu levels increased with dietary Cu supplementation for 19 weeks in a dose-dependent manner. In laying hens, egg Cu was increased by dietary Cu supplementation at a dose of 250 mg/kg for 24 weeks (Pekel and Alp, 2011). The lack of obvious change in egg Cu appears to be related to the relatively shorter experimental period in the present study (4 weeks) and the unchanged Cu intake. The Cu content in the eggshell increased with Cu-treatment, which is thought to result from a relatively higher Cu serum level in the Cu treatment (+21.5%) compared with the control (Table 3). Whether the result positively influences eggshell quality remains to be investigated further.

In the present study, though the fecal Cu concentration was higher in the Cu treatment, the total fecal Cu excretion did not change, indicating that the increased Cu intake via waterline had no obvious influence on Cu excretion and, in turn, can affect the environment. The excreta Cu was significantly increased with dietary Cu supplementation (Pekel and Alp, 2011).

Cu is an integral part of CuZn-SOD, which is involved in the maintenance of redox balance, and catalyzes the formation of highly reactive hydroxyl radicals when in its 'free' form. Dietary Cu ranging from 0.5 mg/kg to 20 mg/kg primarily influences antioxidant capacity in rats (Roughead *et al*., 1999). In broiler chickens, dietary Cu level is associated with the antioxidant capacity (Song *et al*., 2011). In the present study, T-SOD and CuZn-SOD activities and T-AOC did not significantly change, suggesting that the Cu waterline had no obvious influence on the antioxidant ability of hens (Fig. 2). The unchanged serum Cu level may be a possible explanation for the unchanged antioxidant capacity. Similarly, dietary Cu levels from 5 to 15 mg/kg did not influence plasma Cu and liver CuZn-SOD activity (Koh *et al*., 1996). As a biomarker of lipid peroxidation, MDA is increased by stress (Huang *et al*., 2015; Gao *et al*., 2010). In the ileum, the reduced MDA concentration should result from the elevated CuZn-SOD and GSH-PX activities (Fig. 3). Collectively, the present result implies that the Cu waterline has no unexpected effect on the antioxidant capacity.

Gut microbiota plays a vital role in maintaining normal gastrointestinal and normal digestion of nutrients (Gong *et al*., 2021). The richness and diversity of microbial communities can be reflected by single sample diversity (alpha diversity), including two marker indicators, Chao 1 estimator and Shannon index. Chao 1 is indicative of species richness, while the Shannon index represents microbial population diversity. In this study, copper waterline application increased the Chao 1 and Shannon indexes to a certain extent (Fig. 4), indicating that copper waterline application increased the microbial alpha diversity of cecal content. This result implies a novel finding since previous studies on dietary organic copper or inorganic copper addition emphasized the antimicrobial effect of dietary Cu (Fuller *et al*., 1960), but did not fully investigate the effects of copper on microbial diversity in the hindgut of laying hens.

A similar finding was observed in the OTU levels, which are classified via phylogenetic or population genetics to reflect the abundance of bacteria and genera in the sample.

The application of copper waterline significantly increased the number of OTUs at the order, family, and species levels compared to the control ($P<0.05$) but not at the phylum, class, and genus levels, indicating that copper waterline increases the abundance of bacteria at the order, family, and species level (Fig. 4D). The result suggests that hens with copper waterline have richer microbial abundance with an increased proportion of *Methanocorpusculum*, *Paludibacter*, *Elusimicrobium*, *Gracilibacter*, *Syntrophomonas*, and decreased proportion of *Fucobacterium*, *Anaerobiospirillum*, and *Campylobacter* (Fig. 4). The increased proportion of *Methanocorpusculum* was consistent with the previous work by Wu *et al*. (2021), who reported that it was one of the dominant methanogens that encoded various antioxidant enzymes. The decreased abundance of *Anaerobiospirillum* and *Campylobacter*, two potential pathogenic bacteria in poultry (Zhao *et al*., 2019; Sahin *et al*., 2015), indicate that copper waterline is beneficial for intestinal health. The enhancement of microbial abundance is important in maintaining intestinal stability, protecting the host against certain pathogenic and zoonotic organisms (Nava *et al*., 2005), stimulating immune responses (Mead, 2000), and consequently improving overall animal health. Therefore, the changes in the microbial composition, especially the increase in the relative abundance of cecal bacteria, could be responsible for the improvement in egg quality (egg weight) in our study. It has been reported that the alteration in the microbiota, such as *Sphaerochaeta* and *Enorma*, may affect the metabolism of laying hens to improve egg quality, which is in agreement with our findings (Gong *et al*., 2021).

The antibacterial effects of copper waterline might be partly responsible for the changes in the microbial composition of hens. *E. coli* and *Salmonella* are two main pathogenic microorganisms that lead to foodborne illnesses transmitted from poultry products to humans (Pontin *et al*., 2021). When existing water in Cu^{2+} is released from the copper waterline and enters the bacterial cytomembrane, it breaks the enzyme systems and kills the bacteria (Gregor *et al*., 2011). Similarly, antibacterial activity has been reported for *S. enteritidis* as it inhibits biofilm formation (Pontin *et al*., 2021). In this study, the inhibitory effect of copper treatment on *Salmonella* was observed simultaneously with the increased alpha diversity of bacteria in cecum content, suggesting the selectivity of Cu on microorganisms. Xia *et al*. (2004) found that Cu administration could significantly reduce the total pathogenic organism in the gut and have a positive effect on weight gain. Forouzandeh *et al*. (2021) and Villagómez-Estrada *et al*. (2020) reported that the supplementation of Cu could modulate bacterial community by decreasing the abundance of Streptococcaceae, a major pathogen that causes many diseases (Qiao *et al*., 2014). In contrast, Forouzandeh *et al*. (2021) reported an increase in Clostridiaceae, Peptostreptococcaceae, and Enterococcaceae abundance at the family level in $CuSO_4$ treated chicken. Collectively, the increased microbial diversity and inhibited bacterial colonization should be responsible at least partially for the improved feed efficiency of hens fed with Cu waterline.

In conclusion, the result indicates that copper waterline application is beneficial for the performance and gut health of aged laying hens. In addition, it suggests that copper waterline can inhibit the colonization of pathogenic microorganism such as *E. coli* and *Salmonella* and facilitate the enrichment of cecal microbiota diversity, which improves the performance of hens.

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Conflict of Interest

The authors declare that they have no conflict of interest. We are grateful to the reviewers for their valuable comments and suggestions on the paper.

Author Contributions

Ning Ma, Mengze Song, Sheng Li, and Xiaoyan Lin conducted the experiments; Min Liu and Ning Ma processed the experimental data, performed the analysis, drafted the paper and designed the figures; Hongchao Jiao, Xiaojuan Wang, and Jingpeng Zhao were involved in planning and supervision of the work; Hai Lin and Shuhong Sun contributed to the design and implementation of the research, as well as the funding acquirement, project supervision, paper reviewing, and editing. All authors discussed the results and commented on the paper.

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