

Effects of supplemental copper-methionine chelate and copper-soy proteinate on the performance, blood parameters, liver mineral content, and intestinal microflora of broiler chickens

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Primary Audience: Mineral Supplement Manufacturers, Feed Industry Personnel

SUMMARY

In this study, we investigated the effect of dietary supplementation of Cu-Met chelate (Cu-Met) and Cu-soy proteinate (Cu-SP) on the performance, blood parameters, liver mineral content, and intestinal microflora in broiler chickens. A total of 1,008 hatched Ross broiler chickens were randomly assigned to 1 of 6 dietary treatments (T): T1, control; T2, antibiotic (6 ppm of avilamycin); T3, 50 ppm of Cu as Cu-Met; T4, 100 ppm of Cu as Cu-Met; T5, 50 ppm of Cu as Cu-SP; and T6, 100 ppm of Cu as Cu-SP. Each treatment had 3 replicates of 56 birds (28 birds of each sex). During the 4-wk feeding period, the BW increase of birds in the antibiotic treatment was 3.25% and those of birds in the 100 ppm of Cu treatments were 2.67% on average compared with the control group. The production efficiency factor $\{[\text{livability (\%)} \times \text{live weight (kg)/age (d)} \times \text{FCR}] \times 100\}$ was increased by 5.23% for birds in the antibiotic treatment and by 0.7 to 7.8% for birds in Cu treatments, among which the treatment with 100 ppm of Cu as Cu-SP was highest. The red blood cell level, hematocrit level, and mean corpuscular volume of birds in the Cu treatments were lower than were those of birds in the control group. Copper concentration in the liver increased as the level of Cu supplementation increased. The populations of lactobacilli and total bacteria increased, and that of *Escherichia coli* decreased as the level of Cu increased, whereas all microbes, including *Clostridium perfringens*, decreased in the antibiotic treatment.

Key words: antibiotic, broiler, copper-methionine chelate, copper-soy proteinate, intestinal microflora

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DESCRIPTION OF PROBLEM

The ban on subtherapeutic antibiotic use in European Union countries and elsewhere, and recent moves toward the removal or reduced use of these compounds in other countries, has

placed pressure on the feed and poultry industries to look for viable alternatives [1]. Copper has been added to poultry diets as an antimicrobial and growth promoter for many years [2–7]. Copper is an essential element required by poultry and is a component of various intracellular

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and extracellular enzymes, such as cytochrome oxidase, lysyl oxidase, ceruloplasmin, and superoxide dismutase [8]. Copper supplementation at 125 to 250 ppm has been shown to improve the growth rate and FCR in broiler chickens [9, 10] and pigs [11, 12]. High-level Cu supplementation at 125 to 375 ppm also reduces cholesterol levels in the serum and breast muscle [7], and supplementation at 125 to 250 ppm reduces cholesterol levels in the egg yolk [13]. It was also observed that this level of Cu supplementation (125 to 250 ppm) increases Cu concentration in feces [14], which may inhibit the normal fermentation process, and its accumulation in the soil is a cause of environmental concern [15]. Because of this concern, many countries have lowered the maximum dietary Cu level. Some countries have limited the maximum level in pig diets to 75 to 100 ppm [16]. The Association of American Feed Control Officials [17] also limits the maximum use of supplemental Cu to 185 ppm as Cu-citrate. Supplementation of Cu in the form of chelates, complexes, or proteinates has been considered as an organic alternative in animal diets to alleviate these problems by lowering the effective usage level compared with inorganic Cu. Ammerman et al. [18] reported that relative bioavailability estimates of organic Cu sources vary from 88 to 147% of the response of inorganic cupric sulfate, depending on the animal species. Chelated or complex trace elements may improve the bioavailability of minerals for pigs and poultry. These metal-amino acid chelates or complexes furnish trace elements that are more efficiently absorbed from the gut than those provided by inorganic salts [19, 20]. They also provide readily bioavailable amino acids [20, 21]. Some trace minerals in organic form can be utilized better than those from inorganic sources [22–26]. Pesti and Bakalli [13] reported that dietary supplementation of 250 ppm of Cu in the form of sulfate pentahydrate improved egg production, and Lim and Paik [27] reported that egg production increased with supplemental Cu-Met chelate (**Cu-Met**). Methionine is the most commonly used amino acid chelating agent in the preparation of Cu-amino acid chelates [28]. However, Met is a rather expensive ligand agent. Thus, Cu-soy proteinate (**Cu-SP**) was developed to replace Met with soybean digest by using the method of Fe-soy proteinate

production [29, 30]. This study was conducted to compare the effects of supplementation with Cu-Met and Cu-SP on the performance, blood parameters, and small intestinal microflora in broiler chickens.

MATERIALS AND METHODS

Birds, Diets, and Feeding

A total of 1,008 hatched Ross broiler chickens (504 birds of each sex) were randomly assigned to 1 of 6 dietary treatments. Each treatment had 3 replicate pens of 56 birds (28 birds of each sex). Each pen provided a floor area of 2.0 m (width) × 2.4 m (length), and broilers were fed corn- and soy-based mash diets ad libitum for 4 wk. Body weight gain and feed intake were recorded on d 14 for the starter period (0 to 2 wk) and d 28 for the grower period (3 to 4 wk). The weekly FCR was calculated as feed intake (g) divided by BW gain (g). The production efficiency factor (**PEF**) was calculated as {[livability (%) × live weight (kg)/age (d) × FCR] × 100} [31]. The composition of the basal diet used as the control is shown in Table 1. Six dietary treatments were used: T1, control; T2, antibiotic (6 ppm of avilamycin) [32]; T3, **Cu-Met 50** (50 ppm of Cu as Cu-Met); T4, **Cu-Met 100** (100 ppm of Cu as Cu-Met); T5, **Cu-SP 50** (50 ppm of Cu as Cu-SP); and T6, **Cu-SP 100** (100 ppm of Cu as Cu-SP). Avilamycin was used as the positive control because its use as an antibiotic growth promoter is still allowed. Avilamycin, which belongs to the orthosomycin group, is not absorbed in the intestine and is effective against harmful intestinal microflora. The cost of supplementation was approximately 2.5 cents for the antibiotic treatment and 10 cents for the Cu-Met 100 treatment. The amount of extra Met supplied was 0.025% in T3 and 0.05% in T4, from Cu-Met, and the amount of extra CP supplied was 0.028% in T5 and 0.056% in T6, from Cu-SP. Diets and water were provided ad libitum. The brooder temperature was adjusted to 30°C and the barn temperature was maintained at 28°C during the first week and then decreased by 2°C each week. Light was provided for 24 h. All experimental procedures were approved by the Animal Care Committee of Chung-Ang University.

Preparation of Cu Chelates

The Cu-Met was produced by reacting DL-Met and Cu sulfate at a 2:1 molecular ratio, as described previously [27]. The Cu-Met contained approximately 20% Cu. The Cu-SP was produced by reacting soybean digest and Cu sulfate. The soybean digest was made by hydrolyzing 100 g of soybean meal (44% CP) in 500 mL of distilled water with 2 mL of 28% hydrogen peroxide [33] for 2 h. Two milliliters of Alcalase 2.4 L [34] was then added for further hydrolysis at pH 8 and 60°C for 2 h. A Cu solution was prepared by dissolving 100 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 200 mL of distilled water. The soy digest and Cu solution were mixed at room temperature with an additional 50 mL of 50% NaOH as a pH buf-

fer. The precipitate was separated, oven-dried at 30°C for 2 d, and then crushed into powder. The powder was subsequently tested to confirm approximately 17.4% Cu by analysis.

To determine the formation of complexes of Cu-Met and Cu-SP, Fourier-transform infrared (FT-IR) and x-ray diffraction (XRD) spectra were obtained. The FT-IR spectra were measured on a Shimadzu spectrometer [35] with a resolution of 4 cm^{-1} , and XRD spectra were obtained with an x-ray diffractometer [36]. For the XRD measurements, the wavelength of the Mo x-ray was 1.5418 \AA , with a scan range of $5^\circ < 2\theta < 70^\circ$ at 30 mA and 35 kV [37].

Sample Preparation

Approximately 10 g of feed sample was taken from each 25-kg bag. The pooled samples were mixed and divided to make a 200-g representative sample for chemical analysis. At the end of the 4-wk experiment, 8 birds (an equal number of each sex) of median BW from each treatment were killed by cervical dislocation. Immediately after cervical dislocation, blood samples (5 mL each) were collected into EDTA-treated Vacutainer tubes [38] by heart puncture. A 10-cm segment of the upper part of the ileocecal junction was dissected, and approximately 2 g of ileal content was collected into an Eppendorf tube for microbial analysis. Liver samples were thoroughly cleaned in running tap water and dried according to an AOAC method [39].

Chemical Analysis

The general composition of the basal diet was analyzed using AOAC methods [39]. Amino acids composition was analyzed by an Amino Acid Analyzer [40]. Metabolizable energy was calculated based on NRC [41] data. Mineral contents were measured using inductively coupled plasma spectrometry [42] after wet ashing with HNO_3 and HCl [39].

Analyses of Blood Parameters and Serum Immunoglobulin

Blood samples were analyzed using Hemacyte [43]. Serum was obtained by centrifugation for 20 min at $25,000 \times g$ at room temperature and stored in a refrigerator at -15°C until IgG

Table 1. Composition and nutrient content of the broiler diet

Item	Amount
Ingredient, %	
Corn	54.05
Soybean meal	25.71
Animal fat	4.00
Choline chloride	0.15
Premix ¹	0.10
Corn gluten	6.74
Feather meal	1.50
Dicalcium phosphate	2.08
Limestone	1.08
Salt	0.20
L-Lys	0.39
Wheat meal	4.00
Total	100.00
Nutrient content ²	
ME, kcal/kg	3,100
CP, %	21.90
Ca, %	0.91
Available P, %	0.49
Lys, %	1.30
Met, %	0.55
Fe, mg/kg	139.7
Cu, mg/kg	15.3
Zn, mg/kg	86.9

¹Contains the following per kilogram of diet: vitamin A, 12,500,000 IU; vitamin D₃, 2,500,000 IU; vitamin E, 20,000 IU; vitamin K₃, 2,000 mg; vitamin B₁, 2,000 mg; vitamin B₂, 5,000 mg; vitamin B₆, 3,000 mg; vitamin B₁₂, 18 mg; calcium pantothenate, 8,000 mg; folic acid, 1,000 mg; biotin, 50 mg; niacin, 24,000 mg; Zn, 60,000 mg; Mn, 50,000 mg; Fe, 50,000 mg; Cu, 6,000 mg; Co, 250 mg; I, 1,000 mg; Se, 150 mg.

²Analyzed values except for ME, which was calculated based on NRC [41].

and IgA analyses. The IgG and IgA were measured using chicken IgG and IgA ELISA Quantitation Kits [44].

Analysis of Intestinal Microflora

Real-Time PCR Analysis. Genomic DNA was isolated from 2 mg ileal content using an UltraClean Fecal DNA Kit [45]. The DNA was stored at -20°C until use. The colonizations of total bacteria, lactobacilli, *Clostridium perfringens*, and *Escherichia coli* were analyzed using real-time PCR. Genomic DNA extracted from samples were used as templates for PCR amplification using SYBR Green PCR technology [46] and an ABI 7500 Real-Time PCR instrument [47]. Species-specific 16S rRNA primers were used for the *C. perfringens* group [F: 5'-ATGCAAGTCGAGCGA (G/T)G-3', R: 5'-TATGCGGTATTAATCT(C/T)CCTTT-3', where F is forward and R is reverse], the *E. coli* subgroup (F: 5'-GTTAATACCTTGCTCATTGA-3', R: 5'-ACCAGGGTATCTAATCCTGT-3'), and *Lactobacillus* spp. (F: 5'-AGCAGTAGGGAATCTTCCA-3', R: 5'-CACCGCTACACATGGAG-3') [48]. Additionally, Univ-518F (5'-CCAGCAGCCCGCGTAATACG-3') and Univ-800R (5'-TACCAGGGTATCTAATCC-3') were used as universal primers. A quantitative real-time PCR-based method was used to measure specific total bacteria, total lactobacilli, total *E. coli*, and total *C. perfringens* concentrations in the segment of the upper ileocecal junction. All species-specific primers yielded a size of 318 bp for total bacteria-specific amplicons, 341 bp for *Lactobacillus* spp. amplicons, 340 bp for *E. coli* amplicons, and 120 bp for *C. perfringens* amplicons on bands of the respective size. Amplification was performed in a final volume of 20 μL containing 10 μL of $2\times$ SYBR Green PCR Master Mix [49], 2 μL of primer (1 μL of forward and 1 μL of reverse), 1 μL of template, and 7 μL of PCR-grade water. Standard curves were constructed using the PCR products of the 16S rRNA gene of *E. coli* ATCC25922. The DNA concentrations used were 1, 10, 100, and 1,000 $\text{pg}/\mu\text{L}$. Absolute quantification was achieved by using standard curves constructed by the amplification of known amounts of target DNA and following a mathematical model described elsewhere [48, 50].

Statistical Analysis

Data were subjected to ANOVA using the GLM procedure [51]. Significant differences among treatment means were measured using Duncan's multiple range test at $P < 0.01$ or $P < 0.05$ [52]. Orthogonal contrasts were conducted to compare the significantly different means of interest.

RESULTS AND DISCUSSION

The FT-IR and XRD spectra of Cu-Met and Cu-SP are shown in Figures 1 and 2, respectively. The FT-IR spectrum of Cu-Met showed characteristic C=O stretching at $1,616\text{ cm}^{-1}$, whereas free Met generally showed several corresponding peaks around $1,600\text{ cm}^{-1}$. On the other hand, the FT-IR spectrum of soy digest exhibited peaks around $1,650$ and $1,400\text{ cm}^{-1}$, which corresponded to the amide I and amide II absorption bands, respectively. Because the carboxyl groups were potential coordination sites for the metal ions, the changes in the FT-IR absorption peaks in the C=O stretching frequency were monitored as evidence of metal complex formation. The spectral changes in Cu-SP showing additional peaks at the region of 610 cm^{-1} , corresponding to metal-O stretching, were observed. The XRD scattering measurement of the powdered sample provided structural information through the determination of crystallinity. For the Cu-Met and Cu-SP, XRD showed Cu complex formation, as well as the existence of the simple Cu salt. The XRD spectrum of Cu-Met showed high crystallinity with a strong intensity on the spectra. The XRD diffraction pattern of Cu-Met was different from that of the Cu sulfate and clearly showed $\text{Cu}(\text{Met})_2$ complex formation [37]. By comparing the XRD spectra of Cu-Met and Cu-SP, we found that the Cu-SP was not crystalline in nature, probably because of the heterogeneity of the soy proteinate ligand.

Body weight gain, feed intake, feed conversion, and mortality of broiler chickens fed the experimental diets for 4 wk are shown in Table 2. Body weight gain during the starter period (0 to 2 wk) was not significantly different among the groups, but BW gain during the grower period (3 to 4 wk) and over the experimental period as a whole (0 to 4 wk) were significantly ($P < 0.05$) different among treatments. Based on

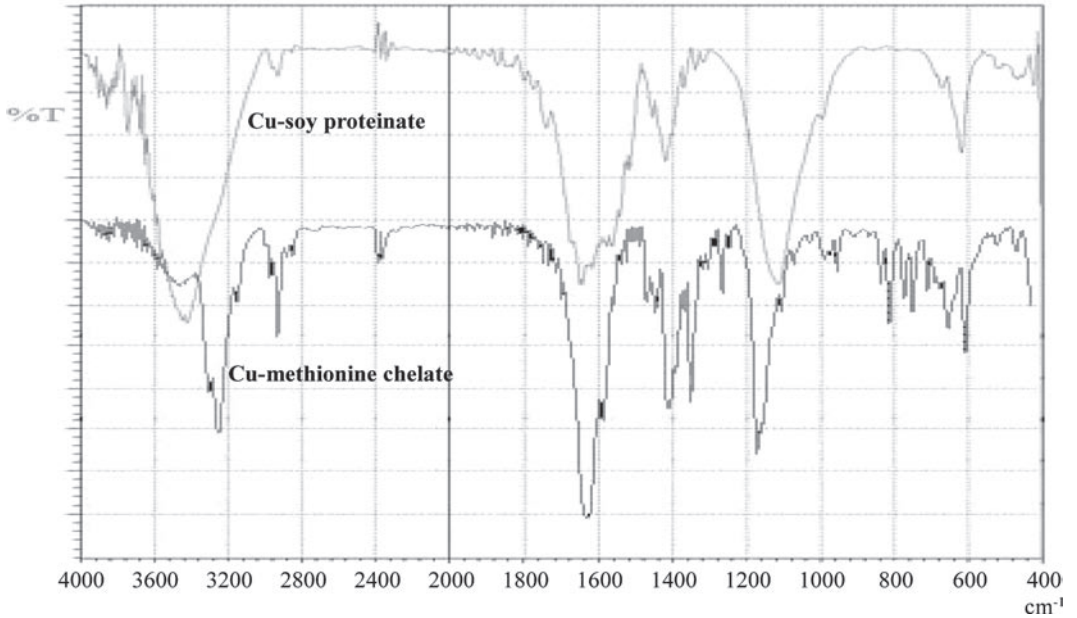


Figure 1. Comparison of Fourier-transform infrared spectra of Cu-Met chelate and Cu-soy proteinate.

orthogonal contrasts, the antibiotic and Cu-chelate treatments resulted in improvements in BW gain by 3.25 and 2.67%, respectively, compared with birds fed the control treatment. In addition, the Cu-Met 100 and Cu-SP 100 treatments resulted in improved BW gain compared with the

Cu-Met 50 and Cu-SP 50 treatments. Within the Cu treatments, the Cu-SP treatments improved BW gain compared with the Cu-Met treatments. Feed intake was significantly ($P < 0.05$) different among treatments during the whole period. The Cu-Met 100 and Cu-SP 100 treatments in-

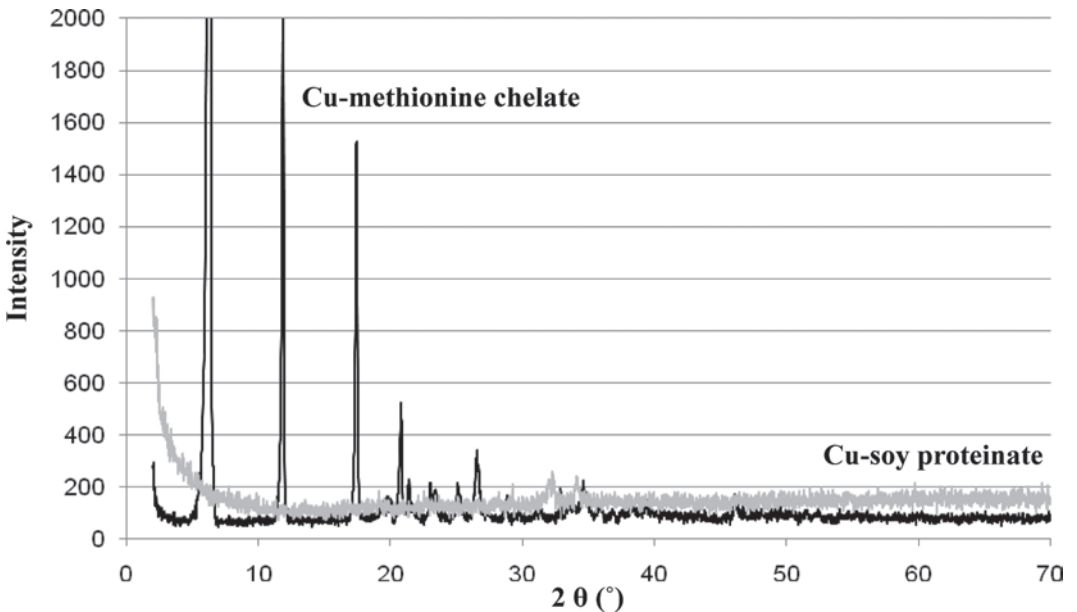


Figure 2. X-ray diffraction spectra of Cu-Met chelate and Cu-soy proteinate.

creased feed intake compared with the Cu-Met 50 and Cu-SP 50 treatments. The FCR was significantly ($P < 0.05$) different among treatments during the starter period. The FCR of the control treatment was lower than those of the other treatments during the starter period. No significant differences were found during the entire period. Mortality was significantly ($P < 0.05$) decreased in the antibiotic and Cu treatments during the starter period, but no significant differences were found over the entire period. There were significant ($P < 0.05$) differences among treatments with respect to PEF. Based on orthogonal contrasts, PEF was improved by 5.23% in the antibiotic treatment and by 0.7 to 7.8% in the Cu treatments. The PEF was highest in the Cu-SP 100 treatment, and the Cu-SP treatments had a higher PEF than the Cu-Met treatments. The levels of leukocytes and erythrocytes in the blood are shown in Table 3. No significant differences were found in leukocyte levels in the blood. However, significant differences were found in red blood cells (RBC), hematocrit (HCT), and mean corpuscular volume (MCV). Supplementation of the antibiotic and Cu products resulted in lower levels of RBC and HCT compared with RBC and HCT in the control treatment. The level of MCV was low in the Cu-supplemented groups compared with that in the control treatment. The levels of IgG and IgA in serum are also shown in Table 3. Concentration of IgA was not significantly different among treatments. The level of IgG in the antibiotic group was significantly ($P < 0.05$) higher than that in the Cu-Met 50 group, and IgG levels in the other treatments were not significantly different. The Cu, Fe, and Zn contents in the livers of birds in the treatment groups are shown in Table 4. Liver concentrations of Fe and Zn were not significantly influenced by treatment. However, significant ($P < 0.01$) differences were found in Cu. Orthogonal contrasts showed that concentration of Cu was higher in the Cu-supplemented treatments than in the control and antibiotic treatments. Orthogonal contrasts in the Cu-Met 100 and Cu-SP 100 treatments were higher than those in the Cu-Met 50 and Cu-SP 50 treatments. The microbial population in the small intestinal segment is shown in Figure 3. Significant ($P < 0.05$) differences were found among treatments. Orthogonal contrasts showed that the populations of total bacte-

ria and lactobacilli were lower in the antibiotic treatment than in the control and Cu treatments. Bacterial populations in the Cu treatments were higher than those in the control treatment, and populations in the Cu-Met 100 and Cu-SP 100 treatments were higher than those in the Cu-Met 50 and Cu-SP 50 treatments. The population of *E. coli* was lowest in the antibiotic treatment, followed by the Cu treatments. The *E. coli* populations in the Cu-SP treatments were lower than those in the Cu-Met treatments, and populations in the Cu-Met 100 and Cu-SP 100 treatments were lower than those in the Cu-Met 50 and Cu-SP 50 treatments. The population of *C. perfringens* in the antibiotic treatment was lower than those in the control and Cu treatments.

Chelation of Cu-Met was reported by Han et al. [37], and the formation of a copper ion complex in Cu-SP was illustrated by Chi and Han [53]. The FT-IR and XRD spectra of Cu-Met and Cu-SP conformed to the data from those studies. This procedure can be used to confirm the chelation of Cu in the quality control laboratory of the related industries.

Supplementing Cu-SP was more effective than supplementing Cu-Met. Improvements in BW gain and feed conversion were reported when broilers were fed diets supplemented with 125 to 250 ppm of Cu in the form of Cu sulfate [9, 54]. The growth performance of broilers and pigs improved with supplementation of chelated Cu and Zn at supranutritional levels [24]. It has also been reported that supplementing 100 ppm of Cu in the form of Met-Cu or Met-Cu-Zn improves performance in broilers [55], and egg production increases with supplementation of 100 ppm of Cu as Cu-Met [27]. Information on the effects of supplemental Cu on blood parameters in poultry is rare. The critical influence of Cu, through ceruloplasmin, in mobilizing absorbed Fe for hemoglobin synthesis has been demonstrated in Cu-depleted and Cu-replete rats [56]. Min et al. [57] reported that ceruloplasmin activity increased significantly in rats, but not in broilers, with supplementation of 200 ppm of Cu. The reason for the low levels of RBC, HCT, and MCV in the Cu treatments, which supplied supranutritional levels of Cu compared with the control treatment, was not elucidated. In the present experiment, the serum IgG level was not significantly different between the control and

Table 2. Body weight gain, feed intake, FE, and mortality of broiler chickens fed the experimental diets for 4 wk

Item	Treatment ¹						SEM ²	P-value
	T1: Control	T2: Antibiotic	T3: Cu-Met 50	T4: Cu-Met 100	T5: Cu-SP 50	T6: Cu-SP 100		
BW gain, g/bird								
0 to 2 wk	370.29	360.02	337.56	357.10	372.86	346.15	14.480	0.518
3 to 4 wk	1,008.54 ^b	1,063.75 ^a	1,012.21 ^b	1,057.40 ^b	1,035.64 ^{ab}	1,070.61 ^a	12.142	0.045
0 to 4 wk	1,378.83 ^c	1,423.77 ⁿ	1,349.77 ^d	1,414.50 ^{ab}	1,408.46 ^b	1,416.82 ^{ab}	13.752	0.030
Feed intake, g/bird								
0 to 2 wk	530.79	543.82	521.92	542.90	543.46	541.90	18.446	0.938
3 to 4 wk	1,575.04	1,617.48	1,562.18	1,606.62	1,592.97	1,602.37	29.428	0.774
0 to 4 wk	2,105.83 ^{bc}	2,161.30 ^a	2,084.31 ^c	2,149.52 ^{ab}	2,136.43 ^{ab}	2,143.72 ^{ab}	16.195	0.043
FCR, feed:gain								
0 to 2 wk	1.43 ^c	1.51 ^{abc}	1.54 ^{ab}	1.52 ^{abc}	1.45 ^{bc}	1.57 ^a	0.028	0.040
3 to 4 wk	1.56	1.52	1.54	1.52	1.54	1.49	0.014	0.136
0 to 4 wk	1.52	1.52	1.54	1.52	1.52	1.51	0.010	0.403
Mortality, %								
0 to 2 wk	3.20 ^a	1.28 ^{ab}	0.00 ^b	1.28 ^{ab}	0.00 ^b	0.00 ^b	0.783	0.049
3 to 4 wk	0.66	1.30	0.00	0.65	1.28	0.00	0.703	0.652
0 to 4 wk	3.84	2.56	0.00	1.92	1.28	0.00	1.167	0.221
Production efficiency factor ³	310.20 ^b	326.49 ^{ab}	312.22 ^b	326.00 ^{ab}	327.44 ^{ab}	334.46 ^a	5.401	0.050

^{a-d}Values with different superscripts in the same row are significantly different ($P < 0.05$). Orthogonal contrasts: BW gain 3 to 4 wk: T1 vs. T2 to T6; T1 vs. T2 to T6; T1 vs. T3 to T6; T3 and T5 vs. T4 and T6. BW gain 0 to 4 wk: T1 vs. T2 to T6; T1 vs. T3 to T6; T2 vs. T3 to T6; T3 and T4 vs. T5 and T6; T3 and T4 vs. T5 and T6 ($P < 0.05$). Feed intake 0 to 4 wk: T3 and T5 vs. T4 and T6 ($P < 0.05$). FCR 0 to 2 wk: T1 vs. T2 to T6; T1 vs. T3 to T6 ($P < 0.05$). Mortality 0 to 4 wk: T1 vs. T2 to T6; T1 vs. T3 to T6 ($P < 0.05$). Production efficiency factor: T1 vs. T2 to T6; T1 vs. T3 to T6; T3 and T4 vs. T5 and T6 ($P < 0.05$).

¹Control = control diet; antibiotic = control diet + 6 ppm of avilamycin; Cu-Met 50 = 50 ppm of Cu as Cu-Met chelate; Cu-Met 100 = 100 ppm of Cu as Cu-Met chelate; Cu-SP 50 = 50 ppm of Cu as Cu-soy proteinate; Cu-SP 100 = 100 ppm of Cu as Cu-soy proteinate.

² $n = 3$.

³Production efficiency factor = [livability (%) × live weight (kg)/age (d) × FCR] × 100.

Table 3. Levels of leukocytes and erythrocytes in blood and immunoglobulins in serum of broiler chickens fed experimental diets

Item	Treatment ¹						SEM ²	P-value
	Control	Antibiotic	Cu-Met 50	Cu-Met 100	Cu-SP 50	Cu-SP 100		
Leukocytes³								
WBC, thousand/ μ L	26.19	24.04	25.16	23.42	24.52	23.98	1.309	0.158
HE, thousand/ μ L	8.99	7.58	8.65	8.96	8.93	8.34	0.443	0.092
LY, thousand/ μ L	11.75	10.33	12.12	10.96	11.00	11.27	0.686	0.089
SI, HE:LY	0.76	0.73	0.72	0.82	0.81	0.74	0.048	0.126
MO, thousand/ μ L	3.01	2.48	2.70	2.84	2.66	2.60	0.177	0.095
EO, thousand/ μ L	1.41	1.09	1.25	1.17	1.14	1.16	0.159	0.102
BA, thousand/ μ L	0.50	0.36	0.41	0.35	0.32	0.33	0.073	0.152
Erythrocytes⁴								
RBC, M/ μ L	3.37 ^a	2.74 ^b	2.84 ^b	3.01 ^{ab}	2.94 ^{ab}	2.86 ^b	0.142	0.031
Hb, d/dL	13.20	11.90	12.36	12.26	12.03	11.90	0.554	0.154
HCT, %	31.30 ^a	26.20 ^{ab}	26.30 ^{ab}	27.00 ^{ab}	23.70 ^b	26.43 ^{ab}	1.618	0.029
MCV, fL	92.23 ^a	93.93 ^a	90.66 ^a	86.40 ^{ab}	87.26 ^{ab}	79.60 ^b	2.896	0.028
MCH, pg	41.96	43.53	40.86	44.70	38.26	41.06	2.252	0.083
MCHC, g/dL	44.13	44.50	46.16	44.36	47.93	48.16	2.252	0.143
Immunoglobulin, mg/mL of serum								
IgG	6.42 ^{ab}	6.67 ^a	6.18 ^b	6.49 ^{ab}	6.49 ^{ab}	6.55 ^{ab}	0.178	0.050
IgA	5.55	5.84	5.67	5.71	5.64	5.77	0.476	0.965

^{a,b}Values with different superscripts in the same row are significantly different ($P < 0.05$).

¹Control = control diet; antibiotic = control diet + 6 ppm of avilamycin; Cu-Met 50 = 50 ppm of Cu as Cu-methionine chelate; Cu-Met 100 = 100 ppm of Cu as Cu-methionine chelate; Cu-SP 50 = 50 ppm of Cu as Cu-soy proteinate; Cu-SP 100 = 100 ppm of Cu as Cu-soy proteinate.

²n = 8.

³Leukocytes: WBC = white blood cells; HE = heterophils; LY = lymphocytes; MO = monocytes; EO = eosinophils; BA = basophils; SI = stress index; heterophils: lymphocytes.

⁴Erythrocytes: RBC = red blood cells; Hb = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

Table 4. Effect of supplemental Cu sources on the Fe, Cu, and Zn contents in broiler livers

Liver mineral	Treatment, ¹ ppm						SEM ²	P-value
	T1: Control	T2: Antibiotic	T3: Cu-Met 50	T4: Cu-Met 100	T5: Cu-SP 50	T6: Cu-SP 100		
Fe	207.21	204.77	203.80	206.63	207.32	206.18	3.622	0.978
Cu	20.02 ^C	20.16 ^C	21.73 ^C	28.82 ^{AB}	24.44 ^{BC}	29.93 ^A	1.765	0.001
Zn	87.19	87.48	85.31	85.72	86.28	86.28	3.165	0.997

^{A-C}Values with different superscripts in the same row are significantly different ($P < 0.01$). Orthogonal contrast: Cu content: T1 vs. T3 to T6, T3 and T5 vs. T4 and T6, $P < 0.01$.

¹DM basis, fat free. Control = control diet; antibiotic = control diet + 6 ppm of avilamycin; Cu-Met 50 = 50 ppm of Cu as Cu-Met chelate; Cu-Met 100 = 100 ppm of Cu as Cu-Met chelate; Cu-SP 50 = 50 ppm of Cu as Cu-soy proteinate; Cu-SP 100 = 100 ppm of Cu as Cu-soy proteinate.

²n = 8.

Cu treatments. Lebacqz-Verheyden et al. [58] reported that IgG makes up the largest portion of chicken serum immunoglobulins, followed by IgM and IgA. In humans, administration of *Lactobacillus*-based probiotics was associated with enhanced specific and nonspecific immune responses [59]. Copper treatments in the form of 50 to 100 ppm of Cu-Met did not influence the level of blood IgG in layers [60], but the combination of Cu-Met and Zn-Met increased blood IgG in broilers [55]. The interaction between Cu and the intestinal microflora, and their subsequent effects on innate immunity, are not in the scope of this experiment. Mineral levels in the liver showed that Cu supplementation increased

the Cu level but that Fe and Zn were not influenced, and Cu-SP increased the Cu level more than did Cu-Met. Organic Cu supplements, especially Cu-SP, are more readily absorbed and accumulated in the liver than inorganic Cu supplements. The effects on intestinal microflora were different for dietary supplementation of the antibiotic and Cu. The antibiotic treatment decreased populations of all bacterial species measured, whereas the Cu treatment increased populations of lactobacilli and decreased populations of *E. coli* and *C. perfringens*. The Cu-Met 100 and Cu-SP 100 treatments were more effective than the Cu-Met 50 and Cu-SP 50 treatments in increasing lactobacilli. In decreasing *E. coli*,

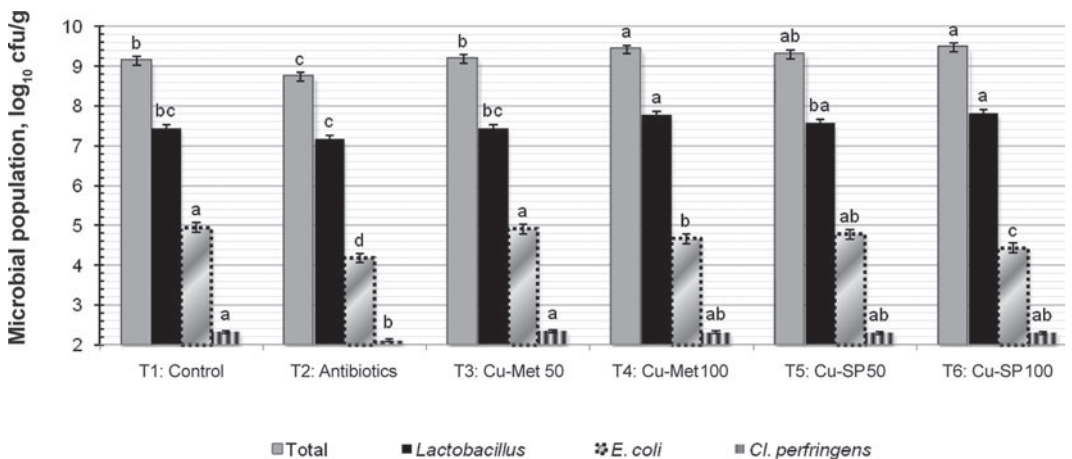


Figure 3. Microbial populations in the small intestinal contents of broiler chickens at 4 wk of age. The colonization of total bacteria, *Clostridium perfringens*, *Escherichia coli*, and lactobacilli in ileal contents was analyzed by real-time PCR. ^{a-d}Treatment means (n = 8) of each microbial population are significantly different at $P < 0.05$. Orthogonal contrasts: Total: treatment (T) 1 vs. T3 to T6; T2 vs. T3 to T6; T3 and T5 vs. T4 and T6 ($P < 0.05$). Lactobacilli: T1 vs. T3 to T6; T2 vs. T3 to T6; T3 and T5 vs. T4 and T6 ($P < 0.05$). *E. coli*: T1 vs. T2 to T6; T1 vs. T3 to T6; T2 vs. T3 to T6; T3 and T4 vs. T5 and T6; T3 and T5 vs. T4 and T6 ($P < 0.05$). *C. perfringens*: T2 vs. T3 to T6 ($P < 0.05$).

the Cu-Met 100 and Cu-SP 100 treatments were more effective than the Cu-Met 50 and Cu-SP 50 treatments, and Cu-SP was more effective than Cu-Met. The antibiotic (avilamycin) suppressed all species of bacteria, as determined with universal primers, whereas Cu did this selectively. Burnell et al. [61] reported that harmful bacteria in the small intestine were inhibited by supplementation with Cu. Hawbaker et al. [62] reported that the growth-promotion effect of Cu in growing pigs may be related to its influence on microbial populations in the digestive tract. Copper decreased *Lactobacillus*, *Streptococcus*, total aerobes, and total anaerobes, and it increased coliforms, molds, and yeast in feces. This response was similar to that of the antibiotic oxytetracycline. However, the pattern of small intestinal microflora in chickens is different from the fecal microflora of pigs. In the present experiment, Cu increased the population of lactobacilli and decreased the populations of *E. coli* and *C. perfringens*. *Lactobacillus* spp. are considered good bacteria, whereas *E. coli* and *C. perfringens* are commensal but are considered harmful because some of these species are pathogenic [63]. Such a pattern in the small intestinal microbial population is similar to that of chickens fed probiotics [64].

Lim and Paik [65] indicated that Cu has a significant influence on the growth regulatory system in many ways. In addition to the effects of dietary Cu on intestinal microflora, Cu reduces the plasma total cholesterol [7, 14]. Zhou et al. [66] also demonstrated that Cu might be involved in pituitary growth hormone gene expression, the secretion of several neuropeptides in the hypothalamus, and the stimulation of growth hormone secretion from bovine pituitary explants. The physiological effects of organic Cu may be more pronounced than those of inorganic Cu when supplemented at the same level. Wedekind et al. [19] and Aoyagi and Baker [20] reported that metal-amino acid chelates or complexes furnish Zn and Cu that are absorbed from the gut more efficiently than those in inorganic form.

In the present experiment, the cost of supplementing the antibiotic was one-fourth the cost of supplementing Cu as Cu-Met 100 (2.5 vs. 10 cents/kg of diet). Therefore, antibiotics will remain as economical growth promoters as long

as their use is allowed in poultry diets. In this experiment, organic Cu supplements used as replacements for antibiotics, especially Cu-SP 100, were comparable with the antibiotic in improving the productivity of broilers. The blood parameters (RBC, HCT, and MCV), Cu level in the liver, and small intestinal microflora were significantly ($P < 0.05$) affected by the treatments.

CONCLUSIONS AND APPLICATIONS

1. The Cu-Met and Cu-SP were made on a pilot scale and their FT-IR and XRD spectra were obtained. This procedure can be used to confirm the chelation of Cu in the related industries.
2. Supplementation of 100 ppm of Cu as Cu-Met or Cu-SP increased BW gain and the PEF of broilers, which were comparable with those from supplementation of the antibiotic (avilamycin).
3. Copper chelates influence the physiological parameters of broilers. They increase the populations of lactobacilli and decrease those of *E. coli* in the intestine.
4. Organic Cu products (Cu-Met and Cu-SP) are potential replacements for antibiotics if the use of subtherapeutic antibiotics is banned on broiler farms and in the feed industry.

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