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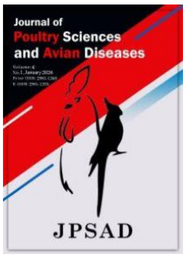

Enhancing Broiler Chicken Growth, Immunological Response, and Intestinal Morphology through Organic Copper Supplementation Combined with Synbiotics

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ABSTRACT

This study was conducted to investigate the effects of organic copper (copper-methionine chelate) in combination with synbiotics (SYN) on the growth performance, morphology, intestinal microbial population, immune response, and meat quality of broiler chickens. 360 mixed-sex, one-day-old broiler chickens were randomly assigned to three levels of organic Cu (8, 16, and 32 mg/kg) and two levels of SYN (0 and 200 mg/kg) in a 3×2 factorial arrangement of treatments, with five replicates of 12 birds each at 6 weeks of age. Interaction effects indicated that from 11 to 24 and 25 to 42 days of age, diets containing higher copper levels (16 and 32 mg/kg) combined with SYN resulted in greater body weight gain compared to diets with 8 or 16 mg/kg of copper without SYN ($p<0.05$). Birds fed a diet containing 8 mg/kg of Cu without SYN exhibited the highest coliform population and pH in the ileum ($p<0.05$). Elevated Cu levels or SYN supplementation improved intestinal morphology, particularly increasing villus surface area and the ratio of villus height to crypt depth. The total antibody titer and IgM in the serum of chickens fed a diet containing 16 mg/kg of Cu along with SYN were significantly higher compared to those fed diets containing 8 and 16 mg/kg of Cu without SYN. Meat analysis (thigh muscle) showed that the percentage of cooking loss in the meat of chickens fed diets containing 32 mg/kg of Cu with SYN was significantly lower compared to chickens fed diets containing 8 mg/kg of Cu without SYN. The inclusion of Cu-methionine chelate alongside SYN significantly improved the performance, morphology, intestinal microbial population, immune response, and meat quality of broiler chickens. These findings provide a basis for the simultaneous application of organic copper and SYN in the diet of broiler chickens.

Keywords: *Broiler chickens, Copper, Growth performance, Gut morphology, Synbiotic.*

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1 Introduction

Copper (Cu) plays a pivotal role in protein synthesis, immune system modulation, and fostering a healthier gut microbiota in broiler chickens (1). Its essential role in growth and immunity highlights its significance in nutrition. Cu, as a growth-promoting additive, can stimulate growth and enhance feed utilization in broiler chickens (2-5). Furthermore, copper significantly contributes to immune response mechanisms and affects gut microbiota by exerting bactericidal or bacteriostatic effects (2, 6, 7).

Inorganic minerals are commonly used in feed to meet animals' needs for trace elements in a cost-effective manner (8). In poultry farming, to prevent trace mineral deficiencies and promote bird growth, these compounds are often used in amounts 2–10 times higher than those recommended by the National Research Council (9). Many studies have confirmed that high concentrations of dietary copper have growth-promoting effects and enhance antioxidant and immune functions (10, 11). However, excessive use may contribute to environmental contamination. To address this, organic minerals like chelates are proposed as substitutes, offering higher bioavailability and potentially allowing lower concentrations in feed without compromising animal performance. Chelates bind with amino acids or peptides, aiding absorption in the intestines. This approach may enhance trace element absorption in the gut by reducing complex formations with fiber, phytates, calcium, and phosphorus, thereby potentially improving mineral utilization efficiency (12). Amino acid-chelated copper can reduce the enthalpy of copper and other nutrients, allowing the mineral to reach its target absorption site without negative interactions with other nutrients in the gastrointestinal tract, thereby promoting the absorption and utilization of copper (13). Cu methionine chelate, an organic Cu compound used in poultry feed, has been shown to improve feed efficiency by reducing Feed Intake (FI) and enhancing performance (14).

In poultry production, antibiotics have historically been used to prevent diseases and enhance growth, but due to heightened concerns about antimicrobial resistance, stricter regulations favoring antibiotic-free practices have emerged (15). Various feed additives, including probiotics, prebiotics, synbiotics (SYN), organic acids, essential oils, and enzymes, have been explored as alternatives to antibiotics (16), demonstrating similar positive effects in modulating gut microbiota and enhancing animal health and growth (17). Supplementing SYN in broiler chickens has demonstrated improvements in body weight, feed efficiency, reduced mortality rates, enhanced heat stress resistance, and lowered levels of harmful gut bacteria (18, 19). These benefits linked to gut health and performance arise from SYN's influence on gut microbial balance (20).

Moreover, the consumption of growth-promoting compounds, such as SYN, prebiotics, and probiotics, has been linked to enhanced mineral absorption and retention in both humans and animals (21, 22). Fermented derivatives of these compounds contribute to improved mineral absorption by reducing gastrointestinal pH, enhancing mineral solubility, producing beneficial bacterial metabolites such as butyrate, stimulating the intestinal epithelium, and increasing absorption capacity (21, 23). Probiotics modify the gut by providing digestive enzymes, reducing pH levels, and increasing enzyme activity (24, 25). At the same time, prebiotics enhance mineral absorption by facilitating water absorption in the colon and lowering intestinal pH, thereby facilitating mineral diffusion (26, 27).

This study aimed to explore the combined effects of Cu methionine chelate and SYN on broiler chickens. It aims to investigate how their concurrent use might synergistically enhance the birds' growth performance, impact their gut health and immune response, and influence carcass components and meat quality.

2 Materials and Methods

2.1 *Management, Birds, and Experimental Design*

A total of 360 one-day-old Ross 308 broiler chickens were randomly assigned to six experimental groups in a 3×2 factorial treatment arrangement. The experimental treatments were as follows: basal diet + 8 mg/kg organic Cu 2. basal diet + 16 mg/kg organic Cu 3. basal diet + 32 mg/kg organic Cu 4. basal diet + 8 mg/kg organic Cu + 200 mg/kg SYN (recommended level) 5. basal diet + 16 mg/kg organic Cu + 200 mg/kg SYN 6. basal diet + 32 mg/kg organic Cu + 200 mg/kg SYN. Factors tested included organic Cu levels (8, 16, and 32 mg/kg of diet) and SYN levels (0 and 200 mg/kg of diet). The experimental treatments were conducted with five replicates of 12 birds each.

Lighting and temperature were regulated according to the management guide for Ross 308 broiler chickens. Feed and water were provided ad libitum to the birds throughout the 42-day experimental period. Broilers were raised in cemented floor pens of identical dimensions (length 120 cm × width 120 cm × height 80 cm) and covered with wood chips. All animal experiments were performed following the guidelines for the care and use of laboratory animals and were approved by the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman (approval number: IR.UK.VETMED.REC.2019-03-05).

2.2 Diets and Supplementation

Basal diets were formulated according to Ross 308 nutrient recommendations (28). Mash diets were formulated for the starter (0-10 days), grower (10-24 days), and finisher (24-42 days) periods (Table 1).

Table 1. Ingredients and composition (as-fed basis) of the basal diets.

Items	Starter diet (d 0 to 10)	Grower diet (d 10 to 24)	Finisher diet (d 24 to 42)
Ingredients (%)			
Corn	56.00	58.25	62.32
Soybean meal	38.00	36.10	31.70
Soybean oil	1.60	1.80	2.20
Dicalcium phosphate	1.75	1.75	1.60
Calcium carbonate	1.00	1.00	1.10
DL-methionine	0.25	0.20	0.15
L- lysine	0.40	0.10	0.13
Threonine	0.20	0	0
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25
Salt	0.30	0.30	0.30
Calculated chemical composition			
Metabolizable energy (Kcal/kg)	2995	3044	3114
Crude protein (%)	22.50	21.90	20.00
Ca (%)	1.00	1.00	0.99
Salt (%)	0.15	0.15	0.15
Available phosphorous (%)	0.45	0.45	0.41
Methionine (%)	0.55	0.51	0.44
Methionine + cysteine (%)	0.99	0.98	0.96
Lysine (%)	1.52	1.26	1.15
Arginine (%)	1.37	1.35	1.21
Threonine (%)	0.98	0.78	0.72

[1] Supplied per kg of diet: vitamin A (retinol), 12000 IU; vitamin D3 (Cholecalciferol), 5000 IU; vitamin K3, 2.55 mg; thiamin, 3 mg; riboflavin, 7.5 mg; vitamin B6 (pyridoxine), 4.5 mg; vitamin B12 (cyanocobalamin), 0.02 mg; niacin, 51 mg; folic acid, 1.5 mg; biotin, 0.2 mg; pantothenic acid, 13.5 mg; choline chloride, 250 mg.

[2] Supplied per kg of diet: Mn, 120 mg; I, 1mg; Se, 0.3 mg; Fe, 40 mg; Zn, 100 mg

The organic Cu (Cu-methionine chelate) was purchased from Ariana Company, Iran. A commercial SYN that contains *Enterococcus faecium*, *Pediococcus acidilactici*, *Bacillus Subtilis*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Saccharomyces cerevisiae*, FOS, and Yeast Extract (lyophilized powder, 3×10^9 CFU/g) was supplied by Zist Darman Mahan company, Bio-Poul® WS, Iran.

2.3 Growth performance

Birds were weighed collectively on days 1, 10, 24, and 42 to ascertain Body Weight Gain (BWG). Feed intake (FI) was computed as the disparity between the feed provided and the feed refused. Mortality was monitored twice daily, and the Feed Conversion Ratio (FCR) was adjusted accordingly to account for mortality.

2.4 Carcass Traits and Internal Organs

At the end of the experimental period, two birds from each cage with a weight comparable to the average weight of the birds in their pen were chosen, weighed, and then decapitated to assess carcass traits and internal organs. The carcass, breast, legs, wings, heart, proventriculus, gizzard, liver, spleen, bursa, and abdominal fat were separated and weighed to calculate their percentage of live weight. Subsequently, the entire left-side thigh muscle was placed in polythene bags and frozen at -20°C for 30 days to evaluate meat quality.

2.5 Intestinal Microbial Population and pH

At the end of the experimental period, two birds from each cage were randomly selected and euthanized by cervical dislocation. Subsequently, the ileal digesta were collected and diluted in phosphate-buffered saline for the enumeration of coliforms (COL) and lactic acid bacteria (LAB). Coliforms were cultured on MacConkey agar at 37°C for 24 hours, while lactic acid bacteria were cultured on MRS agar at 37°C for 72 hours. Dilutions ranging from $10^{\wedge(-2)}$ to $10^{\wedge(-5)}$ were used for coliform counts, and dilutions from $10^{\wedge(-3)}$ to $10^{\wedge(-6)}$ were used for lactic acid bacteria counts (29). The pH of the ileal contents was measured using a digital pH meter (Elmetron, CP103 model) after mixing the digesta with distilled water at a 1:2 ratio.

2.6 Intestinal Morphology

To assess the structure of the ileum tissue, a 1 cm segment of the ileum was isolated and fixed in 10% formaldehyde buffer after washing. Each sample was then embedded in paraffin wax. Hematoxylin and eosin stains were applied to the samples. For evaluating the morphological parameters of the intestine, measurements were taken for villus height, villus width, crypt depth, villus height to crypt depth ratio, villus surface area, epithelial cell layer thickness, and goblet cell density (per 100 µm). The slides were examined using an optical microscope (Micromaster, Fisher Scientific, Cat. No. 12-562-27, Fisher Scientific, Waltham, MA) and analyzed with Image Pro Plus v 4.5 software (Media Cybernetics, Silver Spring, USA) (29).

2.7 Immune Response

To assess the primary and secondary humoral immune response, on days 21 and 35, two birds per replicate were injected brachially with 1 mL of a 0.5% Sheep Red Blood Cells (SRBC) suspension into the brachial vein. Blood samples were collected seven days after injection, and sera were separated by centrifugation (3000 × g, 10 min at 4°C) and stored at -20°C until the antibody titer was measured. The total and IgG anti-SRBC antibodies (mercaptoethanol-resistant antibody against SRBC) were determined using hemagglutination assays conducted in U-bottomed 96-well microplates. The IgM titer was calculated by subtracting the IgG titer from the total titer (29).

2.8 Meat Quality

The lipid oxidation of meat was assessed by quantifying thiobarbituric acid-reactive substances (TBARSs) following the method outlined by Buege and Aust (1978) (30), with modifications as detailed by Khajeh Bami et al. (2021) (31). In summary, 0.5 g of meat was homogenized with 2.5 ml of 0.375% TBA (Sigma-Aldrich, T5500), 0.25 N HCl, and 15% TCA (Merck, k46451107) stock solution. The homogenized meat was then heated in a boiling water bath for 10 minutes and rapidly cooled with tap water. After cooling, the absorbance was measured at 532 nm using a spectrophotometer (Epoch, Biotek, Winooski, VT, USA), and TBARS values were expressed as milligrams of malondialdehyde per kilogram of meat.

The pH values were determined using a digital pH meter following the protocol described by Khajeh Bami et al. (2021) (31). A 5 g sample was homogenized in 25 mL of distilled water for 1 minute before pH measurement.

The water-holding capacity was assessed by placing 1 g of the meat sample on filter paper inside a Falcon tube, centrifuging at 1500 × g for 4 minutes at 4°C, and then drying the samples at 70°C (32). Drip loss was calculated as the percentage of weight lost in the meat after 24 hours of storage at 4°C (33). Cooking loss was determined by measuring the weight difference of meat samples before and after boiling in water for 10 minutes at 85°C (34).

The color parameters (L* = lightness, a* = redness, b* = yellowness) of the meat were assessed at five different locations across the sample surface using digital imaging (31, 35). The hue angle and chroma were calculated as follows: hue angle = tan −1 (a∗/b∗) and chroma = [(a∗) 2 + (b∗) 2] 1/2 (31).

2.9 Statistical Analysis

The data were analyzed using a two-factorial design, considering three levels of organic Cu and two levels of probiotics, utilizing the GLM procedure within Minitab (36). Tukey's test was employed to assess the treatment effects, with significance set at $p<0.05$.

3 Results and Discussion

3.1 Growth Performance

The effects of dietary treatments on growth performance are summarized in Table 2. From 1 to 10 days, 25 to 42 days, and across the entire period (1 to 42 days) of age, broiler chickens fed a diet containing 32 mg/kg of Cu exhibited significantly higher body weight gain (BWG) compared to diets containing 8 mg/kg of Cu (p<0.05). Additionally, broiler chickens fed a diet with 32 mg/kg of Cu demonstrated lower FI and FCR compared to those fed diets containing 8 mg/kg of Cu (p<0.05). Throughout all experimental periods, broiler chickens supplemented with SYN showed greater BWG and lower FI and FCR compared to chickens without SYN supplementation (p<0.05). Interaction effects revealed that from 11 to 24 days of age, the BWG of chickens fed SYN in addition to either 8 mg/kg of Cu or 16 mg/kg of Cu. Those fed 32 mg/kg of Cu without SYN had a significantly higher value than those fed diets containing 8 or 16 mg/kg of Cu without SYN (p < 0.05). Similarly, from 24 to 42 days of age, the BWG of chickens fed 16 or 32 mg/kg of Cu with SYN was significantly higher than that of other experimental groups (p<0.05). Throughout the entire experiment, chickens fed diets containing 16 mg/kg of Cu with SYN exhibited higher FI compared to other treatments, except for birds fed with 8 mg/kg of Cu with SYN (p<0.05). Additionally, broiler chickens fed 32 mg/kg of Cu, with or without SYN, showed a lower FCR compared to chickens fed diets containing 16 mg/kg of Cu with SYN (p<0.05).

Table 2. Effects of Cu-methionine chelate and synbiotics on the growth performance of broilers.

Items	Body weight gain (g/b/d)				Feed intake (g/b/d)				Feed conversion ratio (g/g)			
	d 1 to 10	d 11 to 24	d 24 to 42	d 1 to 42	d 1 to 10	d 11 to 24	d 24 to 42	d 1 to 42	d 1 to 10	d 11 to 24	d 24 to 42	d 1 to 42
Cu level (mg/kg)												
8	12.32^{b}	43.48	71.69^{b}	47.29^{b}	14.09^{a}	61.25	112.8^{a}	71.63^{a}	1.147^{a}	1.399	1.564	1.525
16	12.56^{ab}	43.75	75.20^{a}	49.75^{a}	13.93^{ab}	61.08	114.1^{a}	72.21^{a}	1.111^{b}	1.374	1.530	1.506
32	12.92^{a}	44.20	76.99^{a}	50.28^{a}	13.71^{b}	60.63	110.1^{b}	70.01^{b}	1.062^{c}	1.386	1.503	1.495
SEM	0.124	0.328	0.620	0.461	0.069	0.302	0.583	0.430	0.009	0.015	0.017	0.019
Synbiotics (SYN)												
0 mg/kg	12.19^{b}	42.93^{b}	71.71^{b}	47.97^{b}	13.67^{b}	60.20^{b}	110.4^{b}	70.19^{b}	1.124^{a}	1.399	1.548	1.540^{a}
200 mg/kg	13.01^{a}	44.70^{a}	77.54^{a}	50.24^{a}	14.15^{a}	61.77^{a}	114.3^{a}	72.37^{a}	1.089^{b}	1.374	1.517	1.477^{b}
SEM	0.101	0.262	0.528	0.376	0.056	0.247	0.476	0.351	0.007	0.012	0.014	0.016
Interaction												
8 mg/kg Cu- 0 mg/kg SYN	11.96	42.26^{b}	70.28^{b}	45.91	13.92	60.29	112.2^{bc}	70.98^{bc}	1.168	1.421	1.587	1.574
8 mg/kg Cu - 200 mg/kg SYN	12.68	44.70^{a}	73.10^{b}	48.68	14.25	62.20	113.5^{b}	72.27^{ab}	1.125	1.377	1.541	1.477
16 mg/kg Cu - 0 mg/kg SYN	12.04	41.95^{b}	70.93^{b}	48.36	13.67	60.20	108.9^{c}	70.49^{bc}	1.136	1.400	1.555	1.557
16 mg/kg Cu - 200 mg/kg SYN	13.09	45.56^{a}	79.47^{a}	51.13	14.19	61.95	119.2^{a}	73.93^{a}	1.085	1.348	1.504	1.455
32 mg/kg Cu - 0 mg/kg SYN	12.59	44.57^{a}	73.92^{b}	49.64	13.42	60.10	110.0^{bc}	69.11^{c}	1.068	1.375	1.501	1.490
32 mg/kg Cu - 200 mg/kg SYN	13.26	43.82^{ab}	80.05^{a}	50.92	14.01	61.16	110.3^{bc}	70.90^{bc}	1.057	1.397	1.505	1.500
SEM	0.175	0.437	0.827	0.652	0.098	0.428	0.825	0.608	0.013	0.021	0.026	0.028
p-values												
Cu	0.010	0.319	<0.001	<0.001	0.003	0.350	0.001	0.007	<0.001	0.542	0.068	0.598
SYN	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	0.185	0.149	0.022
Cu × SYN	0.512	<0.001	0.019	0.436	0.433	0.575	<0.001	0.228	0.317	0.200	0.497	0.186

Note: $^{a-c}$ The heterogenous letters in the same column indicate significant differences (p<0.05), and the homogenous letters mean no significant difference (p>0.05)

SEM., standard error of the mean.

Cu has been traditionally used in poultry diets to enhance growth performance and carcass yield (37). Generally, hypotheses regarding the growth-promoting effects of Cu supplementation include the regulation of gut microflora (2), an increase in neuropeptide Y and its mRNA expression level (38), and an enhancement in fat digestion by stimulating the activities of lipase and phospholipase enzymes (39). The gut microbiota are likely sensitive to trace elements such as copper, which is toxic to harmful bacterial populations. Cu exhibits antimicrobial effects against pathogenic bacteria, leading to increased nutrient absorption, protein synthesis, and improved growth process in chickens (6). Additionally, reports suggest that Cu plays a role in stimulating and secreting growth hormones, ultimately resulting in improved performance (40).

Furthermore, it has been reported that Cu supplementation, due to its involvement in the synthesis of hemoglobin, has a stimulatory effect on chicken weight gain

(3). Researchers have reported a positive correlation between the population of LAB, BWG, and FCR (5). In the present experiment, the concurrent use of high levels of Cu-methionine chelate and SYN resulted in improved growth performance, possibly due to the synergistic effects of these substances on enhancing microbial populations and gut morphology.

In agreement with the current research findings, the experimental results demonstrated an improved FCR in birds fed a diet containing 150 mg/kg of Cu hydroxychloride compared to those fed diets containing 15 mg/kg of Cu hydroxychloride (37). Another study using different levels of Cu chelate-methionine (0, 50, 100, 150, and 200 mg/kg) in broiler diets showed that birds receiving Cu chelate-methionine significantly consumed more feed, gained more weight, and had a lower FCR at 1 to 35 days (14).

The significant improvement in BWG observed in broilers fed diets supplemented with SYN in this study is consistent with previous research findings (41). Other research results have shown that incorporating SYN supplements in broiler diets leads to improved body weight, increased BWG, and reduced FCR (42). In another study, the effect of SYN supplementation, which contains *Enterococcus faecium* bacteria and *fructooligosaccharides* as a prebiotic, on the health of broilers was investigated. At the end of the trial (at 35 days), birds fed with SYN supplements showed higher body weight, BWG, and improved FCR compared to the control group (43).

3.2 Carcass Traits and Internal Organs

The relative weights of carcass traits in broiler chickens fed with the experimental treatments are detailed in Table 3. Comparatively, the relative weights of the gizzard and abdominal fat were lower in chickens supplemented with SYN than in those not receiving the SYN supplement ($p<0.05$). Conversely, the relative weight of the spleen was higher in chickens fed with the SYN supplement compared to those without it ($p<0.05$). However, the relative weights of the carcass, breast, legs, wings, heart, proventriculus, liver, and bursa were not significantly affected by the experimental treatments.

Table 3. Effects of Cu-methionine chelate and synbiotics on the carcass traits of broilers at 42 d (percentage of live weight).

Items	Carcass	Breast	Legs	Wings	Heart	Proventriculus	Gizzard	Abdominal fat	Liver	Spleen	Bursa
Cu level (mg/kg)											
8	66.42	29.83	25.33	5.280	0.470	0.314	1.395	0.802	2.039	0.110	0.143
16	67.81	30.13	26.01	5.368	0.488	0.331	1.361	0.733	2.142	0.107	0.155
32	68.57	30.92	26.13	5.363	0.485	0.323	1.277	0.551	2.103	0.121	0.158
SEM	0.671	0.284	0.276	0.103	0.018	0.013	0.056	0.098	0.066	0.005	0.008
Synbiotics (SYN)											
0 mg/kg	66.98	29.84	25.58	5.202	0.481	0.336	1.465^{a}	0.837^{a}	2.118	0.100^{b}	0.138
200 mg/kg	68.22	30.74	26.06	5.472	0.481	0.309	1.223^{b}	0.554^{b}	2.071	0.125^{a}	0.165
SEM	0.517	0.281	0.225	0.084	0.015	0.012	0.044	0.079	0.054	0.004	0.008
Interaction											
8 mg/kg Cu- 0 mg/kg SYN	65.81	29.66	25.31	5.175	0.481	0.309	1.515	0.946	2.051	0.098	0.139
8 mg/kg Cu - 200 mg/kg SYN	67.04	30.00	25.34	5.384	0.458	0.318	1.274	0.658	2.027	0.121	0.147
16 mg/kg Cu - 0 mg/kg SYN	67.11	29.78	25.33	5.070	0.496	0.344	1.528	0.874	2.262	0.097	0.133
16 mg/kg Cu - 200 mg/kg SYN	68.51	30.48	26.71	5.667	0.480	0.318	1.193	0.591	2.022	0.116	0.177
32 mg/kg Cu - 0 mg/kg SYN	68.02	30.10	26.10	5.362	0.465	0.357	1.352	0.690	2.042	0.104	0.144
32 mg/kg Cu - 200 mg/kg SYN	69.12	31.74	26.15	5.363	0.504	0.289	1.202	0.413	2.164	0.139	0.173
SEM	0.775	0.359	0.391	0.145	0.026	0.018	0.069	0.114	0.094	0.006	0.012
p-Values											
Cu	0.276	0.163	0.193	0.800	0.753	0.661	0.455	0.242	0.548	0.194	0.675
SYN	0.267	0.078	0.192	0.065	0.991	0.108	0.017	0.038	0.546	0.004	0.166
Cu × SYN	0.990	0.449	0.203	0.197	0.433	0.210	0.649	0.999	0.177	0.542	0.565

Note: $^{a\text{-}b}$ The heterogenous letters in the same column indicate significant differences ($p<0.05$), and the homogenous letters mean no significant difference ($p>0.05$).

SEM., standard error of the mean.

No specific mechanism has been reported for the reduction of lipid synthesis by prebiotics and probiotics. This could be partly due to the increase in beneficial bacteria, such as LAB, which reduces the activity of acetyl-CoA carboxylase. This enzyme limits the rate of fatty acid synthesis (44, 45). In one experiment, a significant reduction in abdominal fat was observed in 35-day-old broiler chickens that received a notable increase in the level of SYN in their diet (46). In other experiments, the addition of a SYN supplement to the diet of broiler chickens reduced the relative weight of abdominal fat compared to the control group (47). Measuring the weight of immune organs is a common method for assessing the immune status in chicks (48). These organs include the bursa of Fabricius, liver, and spleen, and their optimal growth is crucial for the synthesis of Ig (49). The thymus, spleen, and bursa are integral parts of the avian immune system responsible for producing, differentiating, and replicating immune cells (50). In the current experiment, the weight of the spleen significantly increased in the groups receiving the SYN supplement.

3.3 *Intestinal Microbiota and pH*

The results concerning the effects of different Cu and SYN levels in broiler chicken diets on intestinal microflora and intestinal pH are summarized in Table 4. The findings revealed that the population of COL and pH in the ileum of chickens fed diets with 16 and 32 mg/kg of Cu decreased significantly compared to those fed a diet with 8 mg/kg of Cu ($p < 0.05$). Furthermore, the ratio of LAB to COL in the ileum of chickens fed a diet with 16 mg/kg of Cu was notably higher than in those fed a diet with 8 mg/kg of Cu ($p<0.05$). The inclusion of SYN supplement significantly increased the population of LAB, the ratio of LAB to COL, and reduced COL and pH in the ileum of broiler chickens ($p<0.05$). Interaction effects revealed that chickens fed a diet with 8 mg/kg of Cu without SYN exhibited the highest population of COL compared to other treatments ($p<0.05$). Additionally, the intestinal pH of chickens fed diets with 8 and 16 mg/kg of Cu with or without SYN was significantly lower than in chickens fed diets with 8 mg/kg of Cu without SYN ($p<0.05$).

Table 4. Effects of Cu-methionine chelate and synbiotics on the ileal microflora (log cfu/g) and pH of broilers at 42 d.

Items	Lactic acid bacteria	Coliforms	Lactic acid bacteria /Coliform ratios	Ileal pH
Cu level (mg/kg)				
8	4.786	3.987^{a}	1.285^{b}	6.728^{a}
16	4.988	2.683^{b}	1.728^{a}	6.208^{b}
32	4.784	2.976^{b}	1.657^{ab}	6.131^{b}
SEM	0.116	0.163	0.091	0.029
Synbiotics (SYN)				
0 mg/kg	4.685^{b}	3.478^{a}	1.394^{b}	6.473^{a}
200 mg/kg	5.021^{a}	2.952^{b}	1.719^{a}	6.238^{b}
SEM	0.089	0.144	0.080	0.023
Interaction				
8 mg/kg Cu- 0 mg/kg SYN	4.738	4.714^{a}	1.066	6.795^{a}
8 mg/kg Cu - 200 mg/kg SYN	4.834	3.260^{b}	1.504	6.660^{ab}
16 mg/kg Cu - 0 mg/kg SYN	4.802	2.637^{b}	1.646	6.470^{b}
16 mg/kg Cu - 200 mg/kg SYN	5.173	2.728^{b}	1.810	5.945^{c}
32 mg/kg Cu - 0 mg/kg SYN	4.513	3.084^{b}	1.471	6.152^{c}
32 mg/kg Cu - 200 mg/kg SYN	5.055	2.868^{b}	1.844	6.110^{c}
SEM	0.155	0.231	0.144	0.041
p-Values				
Cu	0.356	<0.001	0.029	<0.001
SYN	0.018	0.021	0.017	<0.001
Cu × SYN	0.409	0.020	0.618	0.006

Note: $^{a\text{-}c}$ The heterogenous letters in the same column indicate significant differences ($p<0.05$), and the homogenous letters mean no significant difference ($p>0.05$).

SEM., standard error of the mean.

Probiotics modify the gut ecosystem by supplying digestive enzymes, reducing pH levels, and enhancing enzyme activity (24, 25). Prebiotics transport water to the cecum through osmotic pressure, enhancing mineral absorption and thereby increasing the volume of fluid in which minerals can dissolve. Moreover, prebiotics, through fermentation, reduce intestinal pH, thereby increasing the concentration of ionized minerals and favoring their inactive dispersion (26, 27). In the present experiment, the simultaneous use of high levels of Cu chelate-methionine and SYN likely enhances nutrient absorption by potentially reducing intestinal pH and increasing enzymatic activity in the digestive system.

In an experiment, adding 100 mg/kg of Cu-methionine chelate to the broiler chicken diet resulted in an increase in LAB and a decrease in *Escherichia coli* (51). Additionally, experimental results showed that adding 30 mg/kg of Cu-methionine chelate to the broiler chicken diet led to an increase in LAB species (52). Synbiotic supplements can modify the gut microbiota by increasing the number of beneficial bacteria and the concentration of lactic acid in the intestines, thereby improving gut health by reducing the count of pathogenic bacteria (53). Fructooligosaccharides present in SYN supplements promote the growth of LAB while significantly inhibiting the prevalence of *E. coli* bacteria.

3.4 *Intestinal Morphology*

The results regarding the morphology of intestinal villi in broiler chickens fed with experimental treatments are summarized in Table 5. Notably, the villus width, ratio of villus height to crypt depth, and villus area were significantly greater in chickens fed a diet with 32 mg/kg of Cu compared to those fed diets with 8 and 16 mg/kg of Cu (p<0.05). Furthermore, the epithelial thickness in the intestines of chickens fed diets with 16 and 32 mg/kg of Cu was notably lower than in those fed a diet with 8 mg/kg of Cu (p<0.05). Regarding SYN supplementation, chickens fed diets containing SYN exhibited significantly higher villus height, ratio of villus height to crypt depth, and villus surface area compared to those without SYN supplementation (p<0.05). Conversely, the crypt depth and epithelial thickness in chickens fed diets with SYN were significantly lower (p<0.05). Interaction effects indicated that the epithelial thickness in the intestines of chickens fed a diet with 8 mg/kg of Cu without SYN was greater compared to other treatments (p<0.05). Moreover, the villus surface area in chickens fed a diet with 32 mg/kg of Cu and SYN was notably greater compared to other treatments (p < 0.05). However, the number of goblet cells was not significantly affected by the experimental treatments in this study.

Table 5. Effects of Cu-methionine chelate and synbiotics on ileal morphology of broilers at 42 d.

Items	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Villus height to crypt depth ratio (μm)	Villus surface area (mm^2)	Epithelial cell layer thickness (μm)	Goblet cell density
Cu level (mg/kg)							
8	726.7	120.3^b	92.97	8.076^b	0.272^b	39.94^a	10.375
16	718.9	131.9^b	89.80	8.131^b	0.302^b	33.63^b	10.375
32	768.3	166.9^a	80.00	9.736^a	0.494^a	33.23^b	11.000
SEM	36.68	1.738	4.779	0.381	0.028	0.886	0.677
Synbiotics (SYN)							
0 mg/kg	677.8^b	126.4	94.58^a	7.269^b	0.251^b	35.94^a	10.000
200 mg/kg	798.2^a	128.4	80.60^b	10.026^a	0.461^a	35.26^b	11.167
SEM	31.57	1.517	3.679	0.311	0.025	0.724	0.583
Interaction							
8 mg/kg Cu- 0 mg/kg SYN	712.8	132.7	103.75	7.060	0.241^b	43.50^a	9.750
8 mg/kg Cu - 200 mg/kg SYN	740.6	131.5	82.20	9.092	0.304^b	36.38^b	11.000
16 mg/kg Cu - 0 mg/kg SYN	659.5	124.7	95.00	6.992	0.236^b	32.25^b	9.750
16 mg/kg Cu - 200 mg/kg SYN	778.4	131.5	84.60	9.270	0.368^b	35.00^b	11.000
32 mg/kg Cu - 0 mg/kg SYN	661.0	122.0	85.00	7.756	0.277^b	32.07^b	10.500
32 mg/kg Cu - 200 mg/kg SYN	875.8	122.1	75.00	11.716	0.711^a	34.40^b	11.500
SEM	48.91	2.628	6.372	0.539	0.037	1.254	0.903
p-Values							
Cu	0.651	0.004	0.160	0.010	<0.001	<0.001	0.779
SYN	0.014	0.390	0.019	<0.001	<0.001	0.513	0.179
Cu × SYN	0.267	0.332	0.631	0.197	0.003	<0.001	0.990

Note: $^{a-b}$The heterogenous letters in the same column indicate significant differences (p<0.05), and the homogenous letters mean no significant difference (p>0.05)

SEM., standard error of the mean

Cu supplements can positively influence the performance of broiler chickens and intestinal physiology. Cu directly stimulates villus regeneration in birds and can indirectly impact gut morphology by influencing the gut microbiota (54). Therefore, in this experiment, the use of higher levels of Cu possibly improved bird growth performance either directly or by enhancing gut microbial populations. In another experiment, adding 30 and 75 mg/kg of Cu-methionine chelate to the diet of broiler chickens resulted in increased villus length and decreased crypt depth (52). In another study, it was demonstrated that adding SYN to the diet of broiler chickens increased villus height and the villus height-to-crypt depth ratio in the gut (55).

3.5 Immune Response

The impact of experimental treatments on the antibody titer against SRBC at 28 and 42 days is outlined in Table 6. Notably, at 42 days, the total antibody titer in the serum of chickens fed diets with 16 and 32 mg/kg of Cu was significantly higher compared to those fed a diet with 8 mg/kg of Cu ($p<0.05$). Similarly, at 28 days, the total antibody titer and IgM in chickens fed with SYN supplements were notably higher than in those without SYN supplementation ($p<0.05$). Interaction effects at 28 days revealed that the total antibody titer and IgM in the serum of chickens fed diets containing 16 mg/kg of Cu with SYN were significantly higher compared to those fed diets with 8 and 16 mg/kg of Cu without SYN ($p<0.05$). These findings highlight the potential synergistic effects of Cu levels and SYN supplementation on antibody titer against SRBC in broiler chickens.

Table 6. Effects of Cu-methionine chelate and synbiotics on the antibody response to sheep red blood cells (log2) broilers at 28 and 42 d.

Items	Total antibody		IgG		IgM	
	d 28	d 42	d 28	d 42	d 28	d 42
Cu level (mg/kg)						
8	3.700	4.000[b]	1.200	1.667	2.500	2.333
16	4.140	5.667[a]	1.500	2.000	2.640	3.667
32	4.300	6.000[a]	1.300	2.500	3.000	3.500
SEM	0.242	0.408	0.158	0.288	0.253	0.384
Synbiotics (SYN)						
0 mg/kg	3.627[b]	5.222	1.400	2.222	2.227[b]	3.000
200 mg/kg	4.467[a]	5.222	1.267	1.889	3.200[a]	3.333
SEM	0.198	0.333	0.129	0.235	0.206	0.314
Interaction						
8 mg/kg Cu- 0 mg/kg SYN	3.400[b]	3.667	1.200	1.667	2.200[b]	2.000
8 mg/kg Cu - 200 mg/kg SYN	4.000[ab]	4.333	1.200	1.667	2.800[ab]	2.667
16 mg/kg Cu - 0 mg/kg SYN	3.080[b]	5.667	1.600	2.333	1.480[b]	3.333
16 mg/kg Cu - 200 mg/kg SYN	5.200[a]	5.667	1.400	1.667	3.800[a]	4.000
32 mg/kg Cu - 0 mg/kg SYN	4.400[ab]	6.333	1.400	2.667	3.000[ab]	3.667
32 mg/kg Cu - 200 mg/kg SYN	4.200[ab]	5.667	1.200	2.333	3.000[ab]	3.333
SEM	0.343	0.577	0.223	0.408	0.358	0.544
p-Values						
Cu	0.215	0.010	0.407	0.164	0.370	0.061
SYN	0.006	1.000	0.472	0.337	0.003	0.468
Cu × SYN	0.008	0.531	0.876	0.723	0.010	0.584

Note: [a-b] The heterogenous letters in the same column indicate significant differences ($p<0.05$), and the homogenous letters mean no significant difference ($p>0.05$).

SEM., standard error of the mean

The experimental results indicated that adding a Cu supplement to the diet of broiler chickens increased the activities of Cu-zinc superoxide dismutase, glutathione peroxidase, and ceruloplasmin in the serum. Furthermore, the Cu supplement led to increased levels of IgA and IgM in the serum (56). In another experiment, broiler chickens fed with a 100 mg/kg of chitosan Cu supplement showed a significant increase in serum IgA and IgM concentrations compared to the control group (1). The study's results demonstrated that adding a Cu supplement to the diet of broiler chickens significantly increased the levels of serum IgM and total antioxidant capacity compared to the control group (57).

Since SYN can be considered an alternative to antibiotics in poultry production, it influences avian health, blood biochemical indices, and the modulation of avian immune system function (58). Research results have shown that the use of SYN supplements in the diet of broiler chickens leads to an increase in the number of immune cells, such as B and T lymphocytes (59). In a study, the addition of a probiotic supplement to the diet of broiler chickens increased the serum IgM concentration (60).

3.6 Meat Quality

The results regarding the meat quality of broiler chickens fed with experimental treatments are presented in Tables 7 and 8. Notably, the water holding capacity, b* value, and chroma in the meat of chickens fed a diet with 32 mg/kg of Cu were significantly higher compared to those fed a diet with 8 mg/kg of Cu ($p<0.05$). Additionally, the percentage of cooking loss and drip loss in the meat of chickens supplemented with SYN was significantly lower compared to treatments without SYN supplementation ($p<0.05$). Interaction effects revealed that the meat cooking loss in chickens fed diets containing 32 mg/kg of Cu with SYN was significantly lower compared to those fed diets with 8 mg/kg of Cu without SYN ($p<0.05$). Furthermore, the TBARS level and chroma value in the meat of chickens fed diets with 32 mg/kg of Cu without SYN and diets with 16 mg/kg of Cu with SYN were notably lower compared to those fed diets with 16 mg/kg of Cu without SYN ($p < 0.05$). Interestingly, the pH of the chicken meat was not significantly affected by the experimental treatments in this study. These findings highlight the potential benefits of Cu levels and SYN supplementation on improving meat quality parameters, such as water-holding capacity, cooking loss, drip loss, TBARS level, and chroma value, in broiler chickens.

Table 7. Effects of Cu-methionine chelate and synbiotics on meat quality parameters of broilers at 42 d.

Items	pH	Water holding capacity (%)	Cooking loss (%)	Driping loss (%)	Thiobarbituric acid reactive substances (ppm malondialdehyde)
Cu level (mg/kg)					
8	6.267	58.88^{b}	32.40	13.98	0.133
16	6.315	61.66^{ab}	32.22	12.83	0.111
32	6.278	63.07^{a}	31.14	13.90	0.087
SEM	0.404	1.009	0.426	0.808	0.013
Synbiotics (SYN)					
0 mg/kg	6.267	61.19	33.33^{a}	14.58^{a}	0.119
200 mg/kg	6.307	61.21	30.52^{b}	12.56^{b}	0.102
SEM	0.033	0.824	0.360	0.660	0.011
Interaction					
8 mg/kg Cu- 0 mg/kg SYN	6.258	57.80	34.98^{a}	15.16	0.137^{ab}
8 mg/kg Cu - 200 mg/kg SYN	6.276	59.96	29.82^{b}	12.80	0.130^{ab}
16 mg/kg Cu - 0 mg/kg SYN	6.284	61.22	32.34^{ab}	12.61	0.149^{a}
16 mg/kg Cu - 200 mg/kg SYN	6.346	62.10	32.11^{ab}	13.04	0.073^{b}
32mg/kg Cu - 0 mg/kg SYN	6.258	64.55	32.67^{ab}	15.98	0.072^{b}
32 mg/kg Cu - 200 mg/kg SYN	6.298	61.58	29.61^{b}	11.83	0.104^{ab}
SEM	0.057	1.428	0.520	1.143	0.021
p-Values					
Cu	0.683	0.023	0.253	0.539	0.081
SYN	0.398	0.984	<0.001	0.040	0.275
Cu × SYN	0.929	0.196	0.010	0.153	0.019

Note: $^{a-b}$ The heterogenous letters in the same column indicate significant differences ($p<0.05$), and the homogenous letters mean no significant difference ($p>0.05$).

SEM., standard error of the mean

Table 8. Effects of Cu-methionine chelate and synbiotics on meat color of broilers at 42 d.

Items	Lightness (L*)	Redness (a*)	Yellowness (b*)	Hue angle (degrees)	Chroma
Cu level (mg/kg)					
8	48.42	25.00	25.38[b]	45.05	36.09[b]
16	46.75	24.50	25.63[ab]	45.12	35.25[b]
32	49.20	25.81	26.99[a]	47.92	37.13[a]
SEM	0.872	0.483	0.398	1.060	0.220
Synbiotics (SYN)					
0 mg/kg	47.83	25.13	25.71	45.01	35.99
200 mg/kg	48.42	25.08	26.29	47.05	36.32
SEM	0.699	0.401	0.331	0.801	0.179
Interaction					
8 mg/kg Cu- 0 mg/kg SYN	47.25	24.00[b]	25.25	45.02	35.37[bc]
8 mg/kg Cu - 200 mg/kg SYN	49.60	26.00[ab]	25.50	45.09	36.81[b]
16 mg/kg Cu - 0 mg/kg SYN	46.25	24.00[b]	25.38	45.19	34.16[c]
16 mg/kg Cu - 200 mg/kg SYN	47.25	25.00[ab]	25.88	45.05	36.34[b]
32 mg/kg Cu - 0 mg/kg SYN	50.00	27.38[a]	26.50	44.82	38.45[a]
32 mg/kg Cu - 200 mg/kg SYN	48.40	24.25[ab]	27.48	51.01	35.81[b]
SEM	1.163	0.720	0.531	1.388	0.311
p-Values					
Cu	0.190	0.210	0.024	0.127	<0.001
SYN	0.587	0.943	0.242	0.116	0.236
Cu × SYN	0.325	0.004	0.815	0.088	<0.001

Note: [a-c] The heterogenous letters in the same column indicate significant differences ($p<0.05$), and the homogenous letters mean no significant difference ($p>0.05$).

SEM., standard error of the mean

Adding 125 mg/kg of Cu to the diet of broiler chickens resulted in a significant reduction in TBARS levels in the meat samples (61). In another experiment, birds fed with Cu supplements showed a decrease in plasma malondialdehyde (MDA) levels (62). SYN can reduce the serum MDA level. This may be due to SYN expressing various enzymes within the antioxidant defense system, thereby inhibiting lipid peroxidation and enhancing antioxidant capacity (63). Several experiments using probiotics and prebiotics have demonstrated a reduction in lipid oxidation in chicken meat, as well as a significant decrease in meat TBARS (64, 65).

In this experiment, the highest water-holding capacity, b* value, and chroma values in chicken meat were observed in birds fed a diet containing 32 mg/kg of Cu compared to those fed a diet containing 8 mg/kg of Cu. The results support that adding Cu sulfate to the diet of broiler chickens increased water-holding capacity in breast and thigh meat (66). In another experiment, adding 10 mg/kg of Cu sulfate to the diet of broiler chickens resulted in increased water-holding capacity in thigh muscles (67). In agreement with this experiment, one study found that increasing the level of SYN in the diet of broiler chickens resulted in a reduction in the percentage of dripping loss and cooking loss in breast and thigh meat (46). In another experiment, adding a SYN supplement to the diet of broiler chickens resulted in a decrease in the percentage of cooking loss in thigh meat (68).

4 Conclusions

Overall, the results of this study demonstrate that the concurrent use of high levels of copper-methionine chelate (16 and 32 mg/kg) and SYN resulted in improved growth performance and immune response. This improvement may be attributed to the synergistic effects of these substances in enhancing microbial populations (LAB and COL) and gut morphology.

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Conflict of Interest

The authors declare no competing interests.

Author Contributions

F.H. contributed to methodology and investigation. M.A. was responsible for conceptualization, methodology, formal analysis, and writing – original draft. M.KB. contributed to methodology, formal analysis, and writing – review and editing.

Data Availability Statement

All data analyzed during this study are included within this article. Any other data is available from the corresponding author upon reasonable request.

Ethical Considerations

All animal experiments were performed under the guidelines for the care and use of laboratory animals and were approved by the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman (approval number: IR.UK.VETMED.REC.2019-03-05).

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