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Original Research Article

Dietary copper requirement of broilers fed a corn-soybean meal diet during 22–42 d of age

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ABSTRACT

This research was to assess the dietary copper (Cu) requirement of broiler chickens fed a practical corn-soybean meal diet during 22–42 d of age. A total of 288 numbered Arbor Acres male broilers at 22 d of age were randomly allotted 6 treatments with 8 replicate cages (6 broilers per cage) per treatment. Broilers were fed a Cu-unsupplemented corn-soybean meal basal diet (control, containing 7.36 mg Cu/kg) or the basal diet added with 3, 6, 9, 12, or 15 mg Cu/kg from $CuSO_4 \cdot 5H_2O$ for 21 d. Quadratic, asymptotic and broken-line models were fitted and the best fitted models were selected to determine dietary Cu requirements. The results revealed that the contents of Cu in serum and liver, mRNA expression levels of Cu- and zinc-containing superoxide dismutase (*CuZnSOD*) in liver and monoamine oxidase b (*MAO B*) in heart, as well as protein expression level of CuZnSOD in liver were affected ($P < 0.05$) by supplemental Cu levels, and the above indices varied linearly and quadratically ($P < 0.05$) with increasing Cu levels. Dietary Cu requirements assessed according to the best fitted broken-line models ($P < 0.05$) of the above indexes were 10.45–13.81 mg/kg. It was concluded that mRNA expression levels of *CuZnSOD* in liver and *MAO B* in heart, as well as liver CuZnSOD protein expression level were new specific sensitive biomarkers for estimating dietary Cu requirements, and the dietary Cu requirement was recommended to be 14 mg/kg to support Cu metabolic needs related to key Cu-containing enzymes in broilers fed the corn-soybean meal diet during 22–42 d of age, which was higher than the dietary Cu requirement (8 mg/kg) for broilers at the corresponding stage suggested by the Chinese Feeding Standard of Chicken.

1. Introduction

As a vital trace element for animals, copper (Cu) is crucial for numerous biological processes in vivo (Balamurugan and Schaffner, 2006; Scheiber et al., 2013). Therefore, it is essential to provide a most suitable Cu level in the diet of broilers to ensure their health and normal growth. The Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004) recommended that dietary Cu requirement for broilers during 1–21 or 22–42 d of age was 8 mg/kg. However, modern broiler breeds have shown a higher growth rate, and thus may have a higher dietary Cu requirement than previous broiler breeds (Applegate and Angel, 2014). As a consequence, it is essential to reassess dietary Cu requirements of modern broiler breeds.

Serum and tissue Cu contents have been considered classical indicators for evaluating dietary Cu requirements of broiler chickens (Lee et al., 2021; Wu et al., 2020). However, the functionality of Cu in the body is primarily dependent on Cu-containing enzymes or proteins, such as Cu- and Zn-containing

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superoxide dismutase (CuZnSOD), monoamine oxidase (MAO), cytochrome *c* oxidase (COX), as well as ceruloplasmin (Linder, 2016; Magour et al., 1979; Ognik et al., 2018; Roychoudhury et al., 2016; Samanta et al., 2011). The CuZnSOD is an antioxidant enzyme whose main function is to scavenge the produced free radicals within the tissues, thus maintaining cellular redox homeostasis (Lewandowski et al., 2018). The MAO is a type of flavoenzyme located in the mitochondrial membrane, which is responsible for catalyzing the oxidation of monoamines to produce the corresponding hydrogen peroxide, aldehydes and ammonia (Wang et al., 2013). The COX is located at the end of the mitochondrion and used for transporting electrons in the respiratory chain to generate molecular oxygen (Ma et al., 2016). Ceruloplasmin belongs to the multi-Cu oxidase family, responsible for the oxidative inactivation of nitric oxide and biogenic amines and acts as antioxidant defense (Hellman and Gitlin, 2002; Linder, 2016). It has also been shown that Cu contents, the activities of key Cu-containing enzymes or the contents of Cu-containing proteins in tissues could be affected by dietary Cu levels in broilers and other animals (da Cruz Ferreira Júnior et al., 2022; Lynch and Strain, 1989; Sharma et al., 2005; Zhang et al., 2009). Recently, the dietary Cu requirement of broilers during 1–21 d of age was studied by our laboratory, and it was found that the kidney MAO activity of chicks fed a corn-soybean meal diet was a new specific sensitive indicator to determine the dietary Cu requirement, which came out at 11.3 mg/kg (Hu et al., 2022). Previous studies demonstrated that when growth performance indices, Cu concentrations in serum and tissues, as well as activities or contents of Cu-containing enzymes or proteins were used as evaluating indicators, dietary requirements for Cu in broilers fed purified or practical diets during 22–42 d of age ranged from 6.75 to 10.75 mg/kg (Li et al., 2014; Shen et al., 1999; Zhou et al., 1996). However, there are following major shortcomings for the above studies on dietary Cu requirements of broilers. Firstly, the use of glucose-soy isolated protein purified diets resulted in a notable decrease in feed intake and growth performance of broilers, and an underestimation of the actual Cu requirement. Secondly, large intervals of added Cu levels caused an inaccurate evaluation of dietary Cu requirements of broiler chickens. Thirdly, evaluating indices used might not be the most sensitive functional indicators because our recent studies have shown that gene expression levels of metalloenzymes or functional proteins were specific and susceptible indicators to evaluate the requirements of iron (Fe), zinc (Zn), manganese (Mn), as well as selenium (Se) in broilers (Huang et al., 2007; Li et al., 2011; Liao et al., 2013, 2017, 2021; Lu et al., 2016; Ma et al., 2016; Wang et al., 2022). Accordingly, it is required to precisely reestimate dietary Cu requirements of modern broiler chickens during 22–42 d of age using practical diets, smaller intervals of added Cu levels and more in-depth molecular indicators. We hypothesized that gene expressions of Cu-containing enzymes or proteins in tissues might be new specific sensitive biomarkers to assess dietary Cu requirement of broilers during 22–42 d of age, and dietary Cu requirement of modern broiler chickens might be higher than the present dietary Cu requirement at the corresponding stage as suggested by the Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004).

In the present study, the effect of dietary supplemental Cu levels on growth performance, Cu concentrations, and gene expressions of key Cu-containing enzymes or functional proteins in serum and different tissues of broilers fed a corn-soybean meal diet during 22–42 d of age was investigated, from which specifically sensitive indices were selected to fit the best mathematical models for accurately evaluating dietary Cu requirements to validate the above hypotheses.

2. Materials and methods

2.1. Animal ethics statement

In the present study, all experimental procedures involving chickens were conducted in compliance with the Animal Experimentation Ethics Committee of Yangzhou University (SYXK (Su) 2021-0027), and adhered to the Experimental Animal Affairs Management Regulations established by the Ministry of Science and Technology in China.

2.2. Animals, diets and experimental design

A total of 350 Arbor Acres male broilers (1-d-old; Jiangsu Jinghai Poultry Company, China) were raised on the equivalent complete corn-soybean meal diet during 1–21 d of age. On d 22, a completely randomized design was employed to randomly allocate 288 broiler chicks with comparable weight to 6 treatment groups (8 replicate cages/treatment and 6 broilers/cage), and raise them in thermostatically measured stainless steel cages (90 cm × 70 cm × 45 cm). The feeding and management of experimental broilers were carried out with the management principles of Arbor Acres broilers. Birds were raised in an environment with continuous 24-h light cycle and they had free access to eat diet and drink tap water containing no detectable Cu during 22–42 d of age. The body weights of some broilers were checked every week end to ensure that their body weights reached the standard body weights, and dead birds were daily recorded. At 42 d of age, the chickens were fasted but allowed to drink tap water overnight. For calculating the average daily gain (ADG), average daily feed intake (ADFI), feed/gain ratio (F:G), and mortality of broilers during 22–42 d of age, the broilers were weighed, and their feed consumptions on the basis of replicate cage were recorded.

According to the recommendations of Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004) and our laboratory's latest result (11.3 mg Cu/kg) on dietary Cu requirement of broilers during 1–21 d of age (Hu et al., 2022), broiler chicks were given a complete corn-soybean meal diet (10.63 mg Cu/kg by analysis) (Table 1) to satisfy all the nutritional requirements. During 22–42 d of age, the broilers in the control group were fed a corn-soybean basal diet without Cu supplement (7.36 mg Cu/kg by analysis, Table 1) to satisfy all other nutritional requirements expect for Cu. The birds in the experimental groups were fed the above basal diet added with 3, 6, 9, 12 and 15 mg Cu/kg in the form of $CuSO_4 \cdot 5H_2O$, respectively, and the measured dietary Cu contents were 7.36, 9.68, 13.46, 16.13, 19.04 and 22.01 mg Cu/kg (Table 2). Broilers were given the meals in mash form.

2.3. Sample collections and preparations

Samples of diets were collected at the right moment for analyzing the contents of calcium (Ca), Cu and crude protein, and the tap water was taken for analyzing Cu concentration. At 42 d of age, 3 chickens from each cage were selected according to the average weight of broilers in cage. Blood was collected from each chicken through wing vein, and then centrifuged at 3,000 × *g* for 15 min to obtain serum for analyzing Cu content, ceruloplasmin concentration as well as CuZnSOD activity. Subsequently, the selected 3 broilers were sacrificed via cervical dislocation, then the tissue samples (heart, liver, kidney, pancreas and spleen) were harvested at once. Liquid nitrogen was used to quickly freeze a portion of tissue samples, after which they were transferred for storage at −80 °C for quantitative analysis of transcription and translation expressions of Cu-containing enzymes or proteins, and another part of tissues were transferred for storage at −20 °C for

Table 1
Composition and nutrient levels of the complete diet for broilers during 1–21 d of age and the basal diet for broilers during 22–42 d of age (as-fed basis).

Item	Day 1–21 (the complete diet)	Day 22–42 (the basal diet)
Ingredients, %		
Corn	53.62	58.34
Soybean meal	37.40	33.24
Soybean oil	4.95	5.04
$CaHPO_4$[1]	1.86	1.52
$CaCO_3$[1]	1.26	1.14
NaCl[1]	0.30	0.30
DL-Met[1]	0.32	0.15
Premix[2]	0.29	0.22
Corn starch + Cu[3]	0	0.05
Total	100	100
Nutrient levels, %		
ME[4], MJ/kg	3035	3095
Crude protein[5]	21.58	20.04
Lys[4]	1.12	1.02
Met[4]	0.61	0.43
L-Thr[4]	0.80	0.74
Trp[4]	0.23	0.21
Met + Cys[4]	0.91	0.72
Ca[5]	0.99	0.92
Nonphytate P[4]	0.45	0.38
Cu[5], mg/kg	10.63	7.36

[1] Feed grade for d 1–21 of age; reagent grade for d 22–42 of age.
[2] Provided per kilogram of diet: vitamin A (as all-trans retinol acetate) 12,000 IU, cholecalciferol 4,500 IU, vitamin E (as all-rac-α-tocopherolacetate) 33 IU, vitamin K_3 (as menadione sodium bisulfate) 3 mg, thiamin (as thiamin mononitrate) 3 mg, vitamin B_2 9.6 mg, vitamin B_6 4.5 mg, vitamin B_{12} 0.03 mg, pantothenic acid calcium 15 mg, niacin 54 mg, folic acid 1.5 mg, biotin 0.15 mg, choline 700 mg, Cu ($CuSO_4 \cdot 5H_2O$) 6 mg, Fe ($FeSO_4 \cdot H_2O$) 40 mg, Zn ($ZnSO_4 \cdot H_2O$) 60 mg, Mn ($MnSO_4 \cdot H_2O$) 110 mg, Se (Na_2SeO_3) 0.35 mg, I ($Ca(IO_3)_2 \cdot H_2O$) 0.35 mg for 1–21 of age, and vitamin A 8,000 IU, cholecalciferol 3,000 IU, vitamin E 22 IU, vitamin K_3 2 mg, thiamin 2 mg, vitamin B_2 6.4 mg, vitamin B_6 3 mg, vitamin B_{12} 0.02 mg, pantothenic acid calcium 10 mg, niacin 36 mg, folic acid 1 mg, biotin 0.1 mg, choline 500 mg, Fe ($FeSO_4 \cdot H_2O$) 30 mg, Zn ($ZnSO_4 \cdot H_2O$) 40 mg, Mn ($MnSO_4 \cdot H_2O$) 80 mg, Se (Na_2SeO_3) 0.35 mg, I ($Ca(IO_3)_2 \cdot H_2O$) 0.35 mg for d 22–42 of age.
[3] $CuSO_4 \cdot 5H_2O$ was supplemented in place of equivalent weights of cornstarch.
[4] Calculated values.
[5] Values were determined by analysis and each value was based on triplicate determinations.

Table 2
Analyzed Cu contents in diets of broilers during 22–42 d of age.

Supplemental Cu, mg/kg	Analyzed Cu contents[1], mg/kg
0	7.36
3	9.68
6	13.46
9	16.13
12	19.04
15	22.01

[1] Values of analyzed Cu contents were based on triplicate determinations.

analyzing Cu content, CuZnSOD and MAO activities. Before analyses, samples from 3 chickens per replicate cage were combined in equal ratios to form one sample.

2.4. Sample analyses

2.4.1. Crude protein, Ca and Cu contents

After wet digestions with HNO_3 and $HClO_4$, Cu contents in the feed ingredient, diet, tap water and tissue samples and Ca contents in feed ingredient and diet samples (500 mg/sample) were measured by the 5110 ICP-OES (Agilent Technologies Australia (M) Pty Ltd., Australia) on the basis of previous description (Huang et al., 2009). The verification of mineral analysis was performed by bovine liver powder (GBW [E] 080193, National Institute of Standards and Technology, Beijing, China). The contents of crude protein in feed ingredient and diet samples (500 mg/sample) were determined using by Association of Official Analytical Chemists (1990) methods. The analysed values are listed in Table 1.

2.4.2. Cu-containing enzymes activities and ceruloplasmin content

A ceruloplasmin assay kit (Jianglaibio Company Ltd., Shanghai, China) was used to determine serum ceruloplasmin content. The CuZnSOD activity in serum as well as CuZnSOD and MAO activities in tissues were measured using commercial kits (Jianchengbio Company Ltd., Nanjing, China). The tissue sample in each treatment group was uniformly weighed to 100 mg for homogenization. Ceruloplasmin content and CuZnSOD activity were determined by a microplate reader (Tecan Experimental Equipment Company, Shanghai, China), and MAO activity was measured by a spectrophotometer (Mapada Instruments, Shanghai, China). The manufacturer's instructions were followed for all assay procedures.

2.4.3. Real-time quantitative PCR (RT-qPCR)

We used TRIzol regent (Vazyme Biological Company Ltd., Nanjing, China) to extract the total RNA from about 50 mg of heart, liver, kidney and pancreas in each treatment group. NanoDrop 2000 spectrophotometer was adopted to detect the RNA concentration. Single-strand cDNA were synthesized using Vazyme Super-Script III Synthesis for RT-PCR kit. RT-qPCR was carried out using the SYBR-Green PCR Master Mix on applied biosystem quantstudio 3D digital PCR System (Applied Biosystems, Foster City, CA). All primer sequence of genes were synthesized by Tsingke Biotechnology (Beijing, China) in Table 3. The method of $2^{-\Delta\Delta CT}$ was used for data analysis, and internal reference genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin, were utilized to standardize the expression levels of the target genes.

2.4.4. Western blotting

The CuZnSOD, MAO A, MAO B, ceruloplasmin, COX 1 and COX 4I1 protein expression levels in liver were measured by the western blotting technique in accordance with the method described by Hu et al. (2022). Total protein in frozen liver samples (100 mg/sample) were obtained by using ice-cold radioimmunoprecipitation assay (RIPA) buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, and the total protein concentration was detected by the Thermo Fisher Scientific BCA protein assay kit. Subsequently, 32 μg of protein samples were loaded and separated on 8% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies were then incubated. A Tanon High-sig ECL Western blotting Substrate Kit was used for display of bands by enhanced chemiluminescence, and the protein expression level was expressed as the intensity of the target gene protein band divided by the intensity of the internal reference GAPDH protein band.

2.5. Statistical analyses

The research of data was analyzed by one-way analysis of variance (one-way ANOVA) using the general linear model procedure of SAS 9.4 (SAS Inst. Inc.). The differences between means were analyzed with the method of least significant difference (LSD). Before analysis, broiler mortality was switched to arcsine. The replication cage composed the experimental unit. The level of dietary Cu supplementation was tested with the linear or quadratic responses of indexes by orthogonal comparisons. And broken-line, quadratic or asymptotic model were used to fit and select the best models, so as to calculate the suitable dietary Cu requirement for broilers (Hu et al., 2022; Liao et al., 2021; Wang et al., 2022). The $P < 0.05$ was regarded as statistically significant.

Table 3
Primer sequences for real-time qPCR amplification.

Genes	GenBank ID	Primer sequences (5′ to 3′)	Product length, bp
Ceruloplasmin	XM_015291853.2	F:GTTATCAAGGCGGAAGTGGG R:TGGGAGGCTGGAGATTCAGTA	158
CuZnSOD	NM_205064.1	F:ATTACCGGCTTGTCTGATGG R:TCCTCCCTTTGCAGTCACAT	174
MAO A	XM_015300467.2	F:GCTCCAAGTTGCTGTATGA R:TCTCAATGCCCAGCTCTTTT	180
MAO B	XM_416766.6	F:ACCAGCTCATCAACCGAATC R:CACAGCCTCGTCTTCCTTTC	247
COX 1	JX_160009.1	F:GCAGGTGTTTCCTCCAT R:GGTTGCGGTCGGTAAGT	187
COX 4I1	XM_015292558.1	F:CTTTCCACCTCCATCTGTGTGA R:GCTGGATGGCTGAAATCG	174
β-Actin	NM_205518.1	F:ACCTGAGCGCAAGTACTCTGTCT R:CATCGTACTCCTGCTTGCTGAT	95
GAPDH	NM_204305.1	F:CTTTGGCATTGTGGAGGGTC R:ACGCTGGGATGATGTTCTGG	128

ID = identity; *CuZnSOD* = Cu- and zinc-containing superoxide dismutase; F = forward; R = reverse; *MAO A* = monoamine oxidase a; *MAO B* = monoamine oxidase b; *COX 1* = cytochrome *c* oxidase subunit 1; *COX 4I1* = cytochrome *c* oxidase subunit 4I1; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

3. Results

3.1. Growth performance and mortality

As shown in Table 4, dietary supplemental Cu level had no effect ($P > 0.05$) on ADG, ADFI, F:G as well as mortality of broilers during 22–42 d of age.

3.2. Cu contents

As shown in Table 5, the dietary added Cu level had no effect ($P \geq 0.05$) on Cu contents in heart, kidney and pancreas of broilers, but affected ($P < 0.01$) Cu concentrations in serum, as well as liver. As the increased dietary Cu levels, serum and liver Cu contents increased linearly and quadratically ($P < 0.05$).

3.3. Enzyme activities and ceruloplasmin content

As shown in Table 6, the added dietary Cu level had no effect ($P > 0.05$) on serum CuZnSOD activity and ceruloplasmin content as well as CuZnSOD and MAO activities in heart, liver, kidney, spleen as well as pancreas.

3.4. mRNA levels in tissues

As shown in Tables 7–10, dietary supplemental Cu levels had no effect ($P > 0.05$) on the mRNA levels of *CuZnSOD* in heart and pancreas, *MAO A* in heart, kidney, liver and pancreas, *MAO B* in kidney and pancreas, ceruloplasmin and *COX 1* in kidney, liver and pancreas, and *COX 4I1* in heart, liver as well as pancreas of broilers. However, mRNA levels of *CuZnSOD* in liver and kidney, *MAO B* in

Table 4
Effect of dietary supplemental Cu level on the growth performance and mortality of broilers during 22–42 d of age[1].

Supplemental Cu, mg/kg	BW on d 21, g	BW on d 42, g	ADG, g/d	ADFI, g/d	F:G, g/g	Mortality[2], %
0	847	2709	88.66	139.8	1.61	4.17
3	847	2619	84.35	141.0	1.67	0.00
6	849	2628	84.69	140.7	1.66	0.00
9	850	2688	87.35	144.0	1.65	0.00
12	847	2594	83.18	136.0	1.66	0.00
15	848	2594	83.24	139.6	1.68	0.00
Pooled SE	6.84	34.04	1.57	2.42	0.01	0.01
P-value						
Added Cu	1.00	0.12	0.11	0.43	0.06	0.09
Linear	0.94	0.05	0.05	0.56	0.01	0.05
Quadratic	0.90	0.90	0.81	0.43	0.28	0.07

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F:G = feed to gain ratio.
[1] Values are the means of 5–8 replicate cages of 6 birds per replicate cage ($n = 5$ to 8).
[2] The percentage of mortality was transformed to arcsine for analysis.

Table 5
Effect of dietary supplemental Cu level on Cu contents in serum and tissues of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	Serum Cu, μg/L	Heart Cu, μg/g, fresh basis	Liver Cu, μg/g, fresh basis	Kidney Cu, μg/g, fresh basis	Pancreas Cu, μg/g, fresh basis
0	71.7^{c}	2.13	2.65^{d}	2.47	1.49
3	111.8^{ab}	2.73	3.54^{c}	2.42	1.51
6	113.5^{a}	2.93	5.94^{a}	2.72	1.54
9	107.4^{ab}	2.87	4.55^{b}	2.57	1.66
12	121.3^{a}	2.82	5.53^{a}	2.46	1.62
15	97.0^{b}	3.17	4.41^{b}	2.61	1.55
Pooled SE	4.68	0.20	0.28	0.10	0.06
P-value					
Added Cu	<0.01	0.05	<0.01	0.32	0.32
Linear	0.01	0.01	<0.01	0.42	0.13
Quadratic	<0.01	0.27	<0.01	0.51	0.18

[a-d] Values with different letter superscripts within the same column differ significantly ($P < 0.05$).
[1] Values are the means of 6 to 8 replicate cages of 3 birds per replicate cage ($n = 6$ to 8).

Table 6
Effect of dietary supplemental Cu level on serum ceruloplasmin content and activities of Cu-containing enzymes in serum and tissues of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	Serum		Heart		Liver		Kidney		Pancreas		Spleen	
	CuZnSOD, U/mg	Ceruloplasmin, μg/mL	CuZnSOD, U/mg prot	MAO, U/mg prot	CuZnSOD, U/mg prot	MAO, U/mg prot	CuZnSOD, U/mg prot	MAO, U/mg prot	CuZnSOD, U/mg prot	MAO, U/mg prot	CuZnSOD, U/mg prot	MAO, U/mg prot
0	320	8.74	410	10.72	292	14.54	200	14.69	75.9	3.03	61.4	14.12
3	377	8.47	354	10.30	301	11.58	190	15.39	77.5	2.85	65.7	12.87
6	330	9.98	360	10.39	272	12.76	190	12.59	76.1	2.64	59.1	15.44
9	322	9.45	355	10.08	308	11.91	186	13.62	85.6	2.60	59.1	14.49
12	295	8.64	326	9.91	319	12.77	196	13.40	76.9	2.59	61.7	14.96
15	337	8.79	355	9.53	309	11.19	185	15.67	85.2	2.71	55.6	13.26
Pooled SE	18.27	1.10	33.87	0.54	14.90	0.95	7.86	0.94	4.45	0.34	7.05	1.10
P-value												
Added Cu	0.10	0.93	0.67	0.74	0.33	0.18	0.73	0.18	0.42	0.94	0.95	0.56
Linear	0.30	0.98	0.21	0.12	0.18	0.08	0.34	1.00	0.16	0.42	0.49	0.91
Quadratic	0.96	0.51	0.37	0.87	0.68	0.52	0.61	0.04	0.92	0.48	0.81	0.30

CuZnSOD = Cu- and Zn-containing superoxide dismutase; MAO = monoamine oxidase.
[1] Values are the means of 7 to 8 replicate cages of 3 birds per replicate cage (n = 7 to 8).

Table 7
Effect of dietary supplemental Cu level on mRNA expression levels of Cu-containing enzymes and ceruloplasmin in heart of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	*CuZnSOD* mRNA, RQ[2]	*MAO A* mRNA, RQ[2]	*MAO B* mRNA, RQ[2]	Ceruloplasmin mRNA, RQ[2]	*COX 1* mRNA, RQ[2]	*COX 4I1* mRNA, RQ[2]
0	1.02	1.01	1.03^{ab}	1.02^{bc}	1.02^{ab}	1.01
3	1.02	1.02	0.72^{c}	1.07^{bc}	1.01^{ab}	0.97
6	0.95	0.98	0.76^{c}	0.95^{c}	0.94^{b}	0.92
9	1.02	0.93	0.92^{bc}	1.14^{abc}	0.88^{b}	0.95
12	1.16	1.00	1.20^{a}	1.24^{ab}	1.16^{a}	1.01
15	1.12	0.93	1.02^{ab}	1.33^{a}	0.89^{b}	0.96
Pooled SE	0.08	0.05	0.07	0.08	0.06	0.05
P-value						
Added Cu	0.51	0.65	<0.01	0.02	0.03	0.78
Linear	0.15	0.24	0.01	<0.01	0.63	0.85
Quadratic	0.41	0.97	0.02	0.15	0.86	0.40

CuZnSOD = Cu- and Zn-containing superoxide dismutase; *MAO A* = monoamine oxidase a; *MAO B* = monoamine oxidase b; *COX 1* = cytochrome *c* oxidase subunit 1; *COX 4I1* = cytochrome *c* oxidase subunit 4I1.
[a-c] Values with different letter superscripts within the same column differ significantly (P < 0.05).
[1] Values are the means of 7 to 8 replicate cages of 3 birds per replicate cage (n = 7 to 8).
[2] The mRNA expression levels were calculated as the relative quantities (RQ) of the target gene mRNA to the geometric mean of β-actin and *GAPDH* mRNA using the $2^{-\triangle\triangle Ct}$ method.

heart and liver, ceruloplasmin and *COX 1* in heart, and *COX 4I1* in kidney were affected (P < 0.05) by dietary Cu supplementation. As the increase of dietary Cu levels, mRNA expression levels of *CuZnSOD* and *COX 4I1* in kidney and ceruloplasmin in heart increased linearly (P < 0.05), while *CuZnSOD* mRNA expression level in liver increased linearly and quadratically (P < 0.05), and *MAO B* mRNA expression level in heart decreased linearly and quadratically (P < 0.05).

3.5. Protein expression levels in liver

Supplemental Cu level had no effect on (P > 0.05) MAO A, MAO B, ceruloplasmin, COX 1, as well as COX 4I1 protein expression levels in the liver (Table 11 and Fig. 1), but CuZnSOD protein expression level in liver was affected (P < 0.01), and increased linearly and quadratically (P < 0.05) with the increase of dietary supplemental Cu levels.

Table 8
Effect of dietary supplemental Cu level on mRNA expression levels of Cu-containing enzymes and ceruloplasmin in liver of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	*CuZnSOD* mRNA, RQ[2]	*MAO A* mRNA, RQ[2]	*MAO B* mRNA, RQ[2]	Ceruloplasmin mRNA, RQ[2]	*COX 1* mRNA, RQ[2]	*COX 4I1* mRNA, RQ[2]
0	1.02^{b}	1.01	1.01^{a}	1.01	1.02	1.02
3	1.01^{b}	1.05	0.78^{bc}	0.93	1.14	1.06
6	1.25^{a}	0.97	0.75^{c}	1.10	0.96	1.06
9	1.18^{ab}	0.89	0.92^{ab}	0.99	1.01	0.99
12	1.21^{a}	0.90	0.88^{abc}	1.00	1.06	1.07
15	1.13^{ab}	1.07	0.79^{bc}	1.00	1.08	1.01
Pooled SE	0.06	0.07	0.05	0.07	0.07	0.07
P-value						
Added Cu	0.02	0.37	0.01	0.75	0.54	0.96
Linear	0.04	0.76	0.21	0.92	0.79	0.88
Quadratic	0.03	0.11	0.19	0.72	0.48	0.73

CuZnSOD = Cu- and Zn-containing superoxide dismutase; *MAO A* = monoamine oxidase a; *MAO B* = monoamine oxidase b; *COX 1* = cytochrome *c* oxidase subunit 1; *COX 4I1* = cytochrome *c* oxidase subunit 4I1.
[a-c] Values with different letter superscripts within the same column differ significantly (P < 0.05).
[1] Values are the means of 6 to 8 replicate cages of 3 birds per replicate cage (n = 6 to 8).
[2] The mRNA expression levels were calculated as the relative quantities (RQ) of the target gene mRNA to the geometric mean of β-actin and *GAPDH* mRNA using the $2^{-\triangle\triangle Ct}$ method.

Table 9
Effect of dietary supplemental Cu level on mRNA expression levels of Cu-containing enzymes and ceruloplasmin in kidney of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	*CuZnSOD* mRNA, RQ[2]	*MAO A* mRNA, RQ[2]	*MAO B* mRNA, RQ[2]	Ceruloplasmin mRNA, RQ[2]	*COX 1* mRNA, RQ[2]	*COX 4I1* mRNA, RQ[2]
0	1.00[b]	1.02	1.02	1.00	1.00	1.00[bc]
3	0.99[b]	0.90	1.01	1.03	0.91	0.97[c]
6	1.08[ab]	0.90	1.01	0.99	0.96	1.17[a]
9	0.94[b]	0.90	0.88	0.90	0.89	1.07[abc]
12	1.16[a]	1.01	1.06	0.90	0.88	1.14[ab]
15	1.16[a]	0.98	1.08	1.07	0.94	1.17[a]
Pooled SE	0.04	0.06	0.09	0.05	0.06	0.05
P-value						
Added Cu	<0.01	0.46	0.79	0.18	0.63	0.03
Linear	<0.01	0.84	0.72	0.69	0.31	0.01
Quadratic	0.18	0.11	0.33	0.10	0.28	0.60

CuZnSOD = Cu- and Zn-containing superoxide dismutase; *MAO A* = monoamine oxidase a; *MAO B* = monoamine oxidase b; *COX 1* = cytochrome *c* oxidase subunit 1; *COX 4I1* = cytochrome *c* oxidase subunit 4I1.
[a-c] Values with different letter superscripts within the same column differ significantly ($P < 0.05$).
[1] Values are the means of 6 to 8 replicate cages of 3 birds per replicate cage ($n = 6$ to 8).
[2] The mRNA expression levels were calculated as the relative quantities (RQ) of the target gene mRNA to the geometric mean of β-actin and *GAPDH* mRNA using the $2^{-\triangle\triangle Ct}$ method.

Table 10
Effect of dietary supplemental Cu level on mRNA expression levels of Cu-containing enzymes and ceruloplasmin in pancreas of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	*CuZnSOD* mRNA, RQ[2]	*MAO A* mRNA, RQ[2]	*MAO B* mRNA, RQ[2]	Ceruloplasmin mRNA, RQ[2]	*COX 1* mRNA, RQ[2]	*COX 4I1* mRNA, RQ[2]
0	1.01	1.02	1.03	1.01	1.02	1.01
3	1.16	1.13	1.08	1.19	1.07	1.02
6	1.02	1.18	1.17	1.08	1.00	0.96
9	1.09	1.09	1.21	1.27	1.02	0.99
12	1.05	1.03	0.94	1.14	0.98	1.03
15	1.14	1.23	1.18	1.09	1.12	1.02
Pooled SE	0.06	0.06	0.09	0.06	0.06	0.05
P-value						
Added Cu	0.42	0.14	0.27	0.08	0.67	0.96
Linear	0.43	0.20	0.61	0.42	0.60	0.91
Quadratic	0.86	0.94	0.56	0.04	0.33	0.54

CuZnSOD = Cu- and Zn-containing superoxide dismutase; *MAO A* = monoamine oxidase a; *MAO B* = monoamine oxidase b; *COX 1* = cytochrome *c* oxidase subunit 1; *COX 4I1* = cytochrome *c* oxidase subunit 4I1.
[1] Values are the means of 6 to 8 replicate cages of 3 birds per replicate cage ($n = 6$ to 8).
[2] The mRNA expression levels were calculated as the relative quantities (RQ) of the target gene mRNA to the geometric mean of β-actin and *GAPDH* mRNA using the $2^{-\triangle\triangle Ct}$ method.

3.6. Estimation of dietary Cu requirements

Dietary Cu requirements of broilers during 22–42 d of age as calculated using the optimal fitted broken-line models ($P < 0.05$) were shown in Table 12. The Cu concentrations in serum and liver, *MAO B* mRNA expression in the heart, *CuZnSOD* the mRNA expression in liver as well as its protein expression level were sensitive biomarkers for evaluating the practical dietary Cu requirements of broilers. According to the best fitted broken-line models of the above sensitive indices, dietary Cu requirements of broilers given a practical corn-soybean meal diet during 22–42 d of age were assessed to be 10.45 to 13.81 mg/kg.

Table 11
Effect of dietary supplemental Cu level on protein expression levels of Cu-containing enzymes and ceruloplasmin in liver of broilers at 42 d of age[1].

Supplemental Cu, mg/kg	CuZnSOD, RQ[2]	MAO A, RQ[2]	MAO B, RQ[2]	Ceruloplasmin, RQ[2]	COX 1, RQ[2]	COX 4I1, RQ[2]
0	0.42[bc]	0.74	0.31	0.77	0.55	0.72
3	0.48[b]	0.76	0.30	0.71	0.50	0.74
6	0.55[a]	0.68	0.26	0.74	0.51	0.74
9	0.42[bc]	0.71	0.25	0.72	0.54	0.70
12	0.42[bc]	0.74	0.25	0.69	0.53	0.78
15	0.38[c]	0.73	0.24	0.74	0.53	0.72
Pooled SE	0.02	0.02	0.02	0.03	0.03	0.03
P-value						
Added Cu	<0.01	0.25	0.15	0.27	0.79	0.44
Linear	0.01	0.51	0.01	0.31	0.85	0.73
Quadratic	<0.01	0.20	0.36	0.19	0.43	0.82

CuZnSOD = Cu- and Zn-containing superoxide dismutase; MAO A = monoamine oxidase a; MAO B = monoamine oxidase b; COX 1 = cytochrome *c* oxidase subunit 1; COX 4I1 = cytochrome *c* oxidase subunit 4I1.
[a-c] Values with different letter superscripts within the same column differ significantly ($P < 0.05$).
[1] Values are the means of 6 to 8 replicate cages of 3 birds per replicate cage ($n = 6$ to 8).
[2] The protein expression levels were calculated as the relative quantities (RQ) of the target gene protein band intensity to the GAPDH protein band intensity.

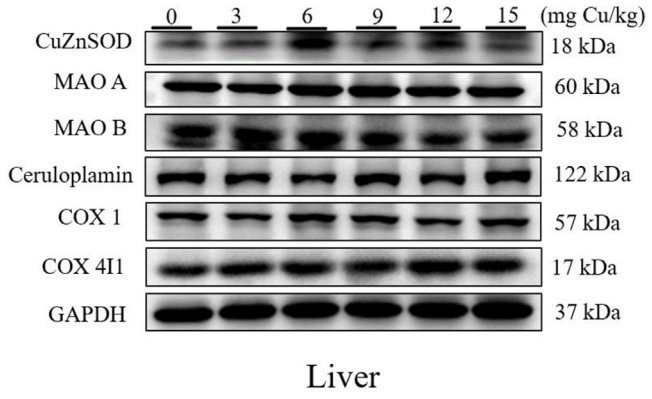

Fig. 1. Representative images of Western blots of Cu-containing enzymes in liver of broilers at 42 d of age. CuZnSOD = Cu- and Zn-containing superoxide dismutase; MAO A = monoamine oxidase a; MAO B = monoamine oxidase b; COX 1 = cytochrome *c* oxidase subunit 1; COX 4I1 = cytochrome *c* oxidase subunit 4I1.

4. Discussion

Here, the experimental data from this study have supported our hypotheses that gene expression levels of Cu-containing enzymes or proteins in tissues might be new specific sensitive biomarkers to assess dietary Cu requirement of broilers during 22–42 d of age, and dietary Cu requirement of modern broilers might be higher than present dietary Cu requirement (8 mg/kg) of broilers at the corresponding stage as suggested by the Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004). Our findings demonstrated that Cu concentrations in serum and liver, mRNA expression levels of *CuZnSOD* in liver and *MAO B* in heart, as well as CuZnSOD protein expression level in liver were sensitive indicators that could be applied for the evaluation of dietary Cu requirements of broiler chickens given the corn-soybean meal diet. Among the above sensitive indicators, heart *MAO B* mRNA expression level, liver *CuZnSOD* mRNA expression level and its protein expression level were new specific sensitive biomarkers for assessing dietary Cu requirements. Furthermore, dietary Cu requirements of broilers during 22–42 d of age were assessed to be 10.45 to 13.81 mg/kg. Therefore, the requirement of dietary Cu was recommended to be 14 mg/kg to support Cu metabolic needs related to key Cu-containing enzymes in broilers given the corn-soybean meal diet during 22–42 d of age, which was higher than the present dietary Cu requirement (8 mg/kg) for broilers at the corresponding stage as suggested by the Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004). These results obtained in our study have been not reported before, and supplied a new insight and scientific basis for fully and accurately meeting dietary Cu requirement of broilers during 22–42 d of age to ensure their health and growth in the broiler production.

In previous studies, dietary Cu requirement of broilers were mainly in accordance with their maximum growth performance parameters (McNaughton and Day, 1979). However, for assessing the Cu requirements of broilers fed with the practical corn-soybean meal diet, the growth performance parameter are often not sufficiently sensitive indicators (Samanta et al., 2011). Herein, our results revealed that supplementation with different levels of Cu had no effect on ADG, ADFI, and F:G of broiler chickens during 22–42 d of age, which was in line with the research results previously reported (Hu et al., 2022; Samanta et al., 2011; Yang et al., 2011), suggesting the Cu content (7.36 mg/kg by analysis) in the basal corn-soymeal diet without Cu supplement and the current Cu requirement specified by the Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004) were sufficient for maintaining the maximum growth performance of broilers.

Increasing evidences have demonstrated that Cu contents in serum, heart as well as liver are commonly used as indicators for assessing Cu status in poultries and ivestocks (Hambidge, 2003; Kang et al., 2007; Wu et al., 2019). It was found that serum Cu content of broiler chicks increased in response to increasing dietary Cu levels (Samanta et al., 2011). Addtionally, it was reported that the level of dietary Cu supplementation increased, and the Cu content in liver of broiler increased linearly (da Cruz Ferreira Júnior et al., 2022). In our previous research on dietary Cu requirement of broilers during 1–21 d of age, we found that serum and liver Cu contents only displayed a linear increase in response to supplemental Cu levels, and thus, they were not appropriative to evaluate dietary Cu requirements (Hu et al., 2022). But intriguingly, the present findings indicated that both the serum and liver Cu contents of broilers increased linearly and quadratically with increasing dietary Cu levels, and thus these two indexes were suitable for assessing dietary Cu requirements of broiler chickens during 22–42 d of age.

Activities, mRNA and protein expression levels of Cu-containing enzymes or functional proteins may be sensitive biomarkers for evaluating Cu status in animals (Failla and Hopkins, 1998; Gomi and Matsuo, 1995; Hambidge, 2003; Harris, 1992). The CuZnSOD is an antioxidant enzyme that catalyzes the conversion of molecular oxygen reduced by single electron into hydrogen peroxide and oxygen, and the catalytic Cu is located at the active center of CuZnSOD to support the normal operation of its enzyme activity

Table 12
Estimation of dietary Cu requirements for broilers during 22–42 d of age based on the best fitted broken-line models.

Dependent variable	Regression equation[1]	R^2	*P*-value	Optimal added Cu level, mg/kg	Dietary Cu requirement[2], mg/kg
Cu content in serum, μg/L	$Y = 71.6701 + 13.3812X$ ($0 \le X \le 3.4890$) $Y = 76.0948 + 12.113X$ ($3.4890 < X \le 15$)	0.5410	<0.01	3.49	10.85
Cu content in liver, μg/g	$Y = 2.5100 + 0.4397X$ ($0 \le X \le 6.00$) $Y = 2.9006 + 0.3746X$ ($6.00 < X \le 15$)	0.5496	<0.01	6.00	13.36
Heart *MAO B* mRNA, RQ	$Y = 1.0288 - 0.1017X$ ($0 \le X \le 3.0863$) $Y = 0.9202 - 0.0665X$ ($3.0863 < X \le 15$)	0.3245	<0.01	3.09	10.45
Liver *CuZnSOD* mRNA, RQ	$Y = 0.9795 + 0.0380X$ ($0 \le X \le 6.4517$) $Y = 1.0373 + 0.0290X$ ($6.4517 < X \le 15$)	0.2177	0.02	6.45	13.81
Liver CuZnSOD protein, RQ	$Y = 0.4200 + 0.0204X$ ($0 \le X \le 5.3170$) $Y = 0.5077 + 0.0039X$ ($5.3170 < X \le 15$)	0.3829	<0.01	5.32	12.68

MAO B = monoamine oxidase b; *CuZnSOD* = Cu- and Zn-containing superoxide dismutase.
[1] The regression equation was fitted according to supplemental Cu levels (0, 3, 6, 9, 12 and 15 mg/kg), where *Y* was the measured index and *X* was the supplemental Cu level (mg/kg) in the basal diet.
[2] Dietary Cu requirement = optimal added Cu level + the Cu content in the basal diet (7.36 mg/kg).

(Scheiber et al., 2013). It was shown that CuZnSOD was a sensitive indicator for evaluating the Zn requirement of Chinese yellow-feathered broilers (Li et al., 2019). Lai et al. (1994) discovered that dietary Cu deficiency could reduce the transcriptional and translational levels of CuZnSOD in the liver of rat fed the Cu-deficient diet for 4 weeks. Furthermore, an earlier investigation demonstrated that liver *CuZnSOD* mRNA expression level of Cobb 500 broilers fed the semi-purified diet at d 17 increased quadratically with increasing levels of dietary Cu supplementation (da Cruz Ferreira Júnior et al., 2022). Our prior research implied that dietary Cu levels did not affect the *CuZnSOD* mRNA and protein expressions in tissues of broiler chicks at d 21 (Hu et al., 2022). However, in this study, we observed that the mRNA and protein expression of *CuZnSOD* in the liver of broilers at 42 d of age increased linearly and quadratically with the increase of dietary Cu levels. This finding may be attributed to the fact that the liver is the main storage and action site of Cu, and CuZnSOD in the liver is more sensitive to the dietary Cu concentration. Therefore, the *CuZnSOD* mRNA and its protein expression levels in the liver were sensitive indicators to evaluate Cu requirements of broiler chickens during 22–42 d of age, and the optimal CuZnSOD expression is beneficial for improving the antioxidant capacity of broilers.

The MAO is a Cu-dependent enzyme with catalytic activity, and it catalyzes the oxidative deamination of monoamines in the body with flavin adenine dinucleotide (FAD) as a cofactor, producing harmful substances such as hydrogen peroxide (Gong and Boor, 2006; Wang et al., 2013). Both MAO A and MAO B are two subtypes of MAO, and they are encoded by different genes as well as differ in sensitivity to inhibitors and substrate specificity (Gong and Boor, 2006). Fu et al. (2020) discovered that dietary Cu levels had an effect on the liver *MAO B* mRNA expression of dairy cows, and the liver *MAO B* mRNA expression decreased linearly with increasing dietary Cu levels. Our previous studies suggested that as the increase of the added Cu level, kidney MAO activity decreased quadratically and was a sensitive index for estimating the dietary Cu requirement of broilers during 1–21 d of age (Hu et al., 2022). In the current study, although MAO activities in tissues did not change, the mRNA expression level of *MAO B* in heart decreased linearly and quadratically with increasing dietary added Cu levels, indicating that its mRNA expression level in heart was a sensitive index for estimating dietary Cu requirement of broiler chickens during 22–42 d of age.

As the key member of multi-Cu oxidase family, ceruloplasmin can carry and transport Cu in plasma and is mainly involved in antioxidant defense outside the cell by oxidizing and inactivating harmful substances such as nitric oxide and biogenic amines (Linder, 2016; Vasilyev, 2019). The COX is a mitochondrial Cu-containing metalloenzyme whose catalytic core site consists of three mitochondria-encoded subunits containing three Cu atoms, and it has a significant influence on the oxidative production of cellular energy and the scavenging of free radicals in mitochondria (Horn and Barrientos, 2008). Both COX 1 and COX 4I1 are two important regulatory subunits of COX. The main role of COX 1 is to indirectly protect various tissues from injury, reduce inflammation and maintain their normal function by synthesizing prostaglandins, and COX 4I1 is regarded as the most important regulatory subunit of COX because its indirect product, adenosine triphosphate, is required for metastable feedback inhibition of the activity of COX (Van der Schueren et al., 2015; Vane et al., 1998). Our results showed that dietary supplemental Cu level affected mRNA expression levels of ceruloplasmin and *COX 1* in heart, and *COX 4I1* in kidney, but had no effect on serum ceruloplasmin content, and gene expressions of ceruloplasmin, COX 1 as well as COX 4I1 in other tissues. Meanwhile, mRNA expressions of *COX 4I1* in kidney, and ceruloplasmin in heart increased linearly with the increase of supplemental Cu levels. Our laboratory previous study indicated that the marginal Cu deficiency in corn-soybean meal diet had no effect on serum ceruloplasmin content, ceruloplasmin and COX expressions in tissues of broiler chicks during 1–21 d of age (Hu et al., 2022). Here, the analyzed result of the Cu content in the corn-soybean meal basal diet without Cu supplement was 7.36 mg/kg, which is close to the current Cu requirement of 8 mg/kg for broiler chickens. Consequently, it was understandable that serum ceruloplasmin content, ceruloplasmin, COX 1 and COX 4I1 gene expressions in tissues of broilers were not affected with the increase of dietary Cu levels. These results indicate that ceruloplasmin content in the serum, ceruloplasmin, COX gene expression in tissues were not suitable to assess the Cu requirement of broiler chickens during 22–42 d of age. The above new findings would be helpful to improve the antioxidant capacity of broiler tissues and provide scientific experimental basis for ensuring adequate Cu supplementation in the broiler production.

5. Conclusion

The findings from our research indicated that mRNA expression levels of *MAO B* in heart and *CuZnSOD* in liver, as well as CuZnSOD protein expression level in liver were new specific sensitive biomarkers for evaluating dietary Cu requirements, and dietary Cu requirement of broiler chickens fed a corn-soybean meal diet during 22–42 d of age was recommended to be 14 mg/kg, which was higher than the present Cu requirement (8 mg/kg) recommended by Chinese Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004) at the corresponding stage.

Author contributions

Ling Zhu: Conceptualization, Methodology, Software, Investigation, Writing - Original Draft. **Wei Wu, Bingxin Wu**, **Liyang Zhang**, **Weiyun Zhang**, **Tingting Li**, **Xiaoyan Cui**, **Feiyu Gao, Ding Li:** Validation, Formal analysis, Software. **Xugang Luo:** Writing - Review & Editing. **Shengchen Wang:** Resources, Writing - Review & Editing, Supervision, Data Curation.

Declaration of competing interest

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

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