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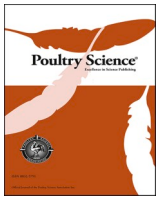

Full-Length Article

The potential of supplementing compound organic trace elements at lower levels in Chinese yellow- feathered broiler diets, part II: Impacts on growth performance, gut health, intestinal microbiota, and fecal mineral excretion

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ABSTRACT

This study aimed to investigate the effects of reducing inorganic trace minerals (**ITM**) by supplementing compound organic trace minerals (**OTM**) chelates on growth performance, fecal mineral excretion, intestinal health, and cecal microbiota of yellow-feathered broilers. A total of 960 one day old male broilers were randomly assigned to 6 treatments, among which birds were fed with the basal diets (negative control, NC), or supplemented with 1,000 mg/kg (positive control, PC), 300, and 500 mg/kg ITM or OTM, respectively. Dietary supplementation of OTM significantly increased the average daily gain (**ADG**) during 22-53 d and 1-53 d, and reduced the fecal emissions of Fe, Cu, Zn, and Mn of Chinese yellow-feathered broilers ($P < 0.05$). Furthermore, the OTM300 group significantly reduced the crypt depth in the duodenum, and increased the ratio of villus height to crypt depth (**V/C**) in the duodenum and jejunum ($P < 0.05$). The mRNA expression of TGF-β, Bcl-2, CAT, and GPX4 as well as tight junction proteins (occludin, ZO-1, claudin-1, and claudin-5) in jejunum mucosa were significantly increased by compound OTM when comparing with ITM300 group ($P < 0.05$). Moreover, dietary compound OTM significantly changed the Chao1 index and β diversity index of cecal microbiota of Chinese yellow-feathered broilers. The abundances of Firmicutes (phylum), *Eubacterium_coprostanoligenes_group* (family) and *Oscillibacter* (genus) were increased, while the abundances of Bacteroidetes (phylum) and *Rikenellaceae RC9 group* (genus) were decreased by OTM treatment. Spearman correlation analysis showed that the mRNA of occludin and jejunal V/C ratio were positively correlated with the abundance of Firmicutes (phylum), but negatively correlated with the abundance of Bacteroidota (phylum). In addition, the abundance of *Eubacterium_coprostanoligenes_group* (family) was positively correlated with the mRNA of claudin-1, Bcl-2, and TGF-β. PICRUST prediction of microbial function revealed that OTM treatment enriched the pathways related to amino acid metabolism and DNA replication. In conclusion, dietary supplementation at lower levels of compound OTM to replace ITM could improve growth performance and intestinal health, and reduce the fecal excretion of trace elements by modulation of cecal microbiota community and diversity in Chinese yellow-feathered broilers.

Introduction

Essential trace elements such as Fe, Zn, Mn, and Cu are critically important for maintaining the normal physiological and nutritional functions of animals, including enzyme function, immune response, and oxidative stress management (Kawahara et al., 2023). For decades, trace element additives have been commonly supplied via the forms of inorganic salts. However, the supplementation amounts of inorganic trace minerals (**ITM**) are often higher than the nutrient recommendation in order to prevent mineral deficiencies or support animal optimal growth due to their easy degradation and antagonism between trace elements, which would lead to environmental pollution by excessive use of ITM.

Previous studies have found that dietary supplementation of

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inorganic source of Zn and Cu could cause antagonistic effects, as reflected by impaired absorption and utilization ITM and other nutrients (Qiu et al., 2023). Supplementation of organic chelated Zn and Cu proteinate could eliminate such disadvantages and improve the bioavailability of trace elements, thereby improving the growth performance and mineral status of chicks (Ao et al., 2009). Furthermore, dietary replacement of ITM with lower levels of organic trace minerals (**OTM**) has been shown to effectively enhance the antioxidant capacity of broilers (Aksu et al., 2010), and significantly reduce fecal mineral excretions in growing-finishing pigs (Xiong et al., 2023), late-phase laying hens (Yenice et al., 2015), as well as 817 white-feathered broilers (Kong et al., 2022) without compromising the normal growth performance. Notably, gut microbiota regulates the metabolism and transport of micronutrients and promotes the bioavailability of trace metals by competing with hosts by absorbing trace metals from food sources (Yu et al., 2022). Recent studies have found that dietary trace mineral patterns could influence the gut microbiota and intestinal health of Arbor Acres broilers (Wang et al., 2023a). Chinese local yellow-feathered broilers have superior meat flavor and taste and stronger disease resistance ability than white-feathered broilers, and accounted for approximately 32% of chicken production in China (FAS GAIN/USDA, 2020). However, it remained largely unknown concerning the effects of amino acids-chelated Fe, Zn, Mn, and Cu in organic sources on performance, gut health, and fecal excretion in Chinese yellow-feathered broilers. Thus, the objective of this study was to investigate the substitution of amino acids-chelated compound OTM of Fe, Zn, Mn, and Cu at lower levels on growth performance, fecal mineral excretion, intestinal health, and cecal microbiota in Chinese yellow-feathered broilers.

Materials and methods

Animal management, diets, and experimental design

All animals used in this study were cared for and handled in accordance with the guidelines of the Institutional Animal Care and Use Committee of Foshan University (FOSU2022004). A total of 960 one day old healthy male broilers were provided from a commercial hatchery (Guangzhou Muyuan Poultry Industry Co., Ltd, China) and were randomly allocated to 6 treatment groups with 8 replicates each and 20 birds per replicate. Birds were raised in cages (65 × 60 × 40 cm) and water and feed were provided ad libitum throughout the trial. The lighting program was 23 h of light and 1 h of darkness from 0 to 7 d of age, followed by 16 h of light for the remainder of the trial. The temperature of the room was maintained at 32 to 34°C for the first 3 d and then reduced by 2 to 3°C per week to a final temperature at 26°C from d 1 to 21, and the temperature was the same for d 22 to 53.

The broilers in the treatment groups were fed with diets as follows: 1) control diet without ITM or OTM premix (negative control, NC); 2) control diet supplemented with ITM premix at 1,000 mg/kg of feed (positive control, PC) with Fe, Cu, Zn, Mn providing commercially recommended concentrations; 3) control diet supplemented with ITM premix at 300 mg/kg of feed (ITM300); 4) control diet supplemented with ITM premix at 500 mg/kg of feed (ITM500); 5) control diet supplemented with OTM premix at 300 mg/kg of feed (OTM300); 6) control diet supplemented with OTM premix at 500 mg/kg of feed (OTM500). In addition, the basal diets, experimental design, and measured values of trace minerals in experimental diets were described in Part I of this study (Nie et al., 2024). The OTM500 groups of Zn, Fe, Cu, and Mn in the basal diets were measured as 78.97, 265.29, 8.26, and 99.65 mg/kg at the starter stage.

Sample collection and measurements

Fresh feces were collected from each replicate for 2 consecutive days before the trial ended and stored at −20°C for further analysis. On the morning of d 53 following an overnight fast, two broilers with similar body weight to the average body weight (BW) from each replicate (n = 8) was randomly selected for sample collections. The middle sections (about 3 cm) of the duodenum, jejunum, and ileum were collected and placed in 4% formalin fixation to determine the intestinal morphology. Then, the mucosa samples were scraped gently from the middle portion of the jejunum after flushing with ice-cold saline, and were collected into 1.5 mL EP tubes and frozen at −80°C for the determination of intestinal cytokines. The contents of the cecum were harvested, snap-frozen in liquid nitrogen, and then stored at −80°C until 16 S rRNA sequencing analysis.

Performance parameters

All broilers were weighed per pen (replicate) at 0, 21, and 53 d, and the average individual BW per bird and average body weight gain (**ADG**) were calculated. Cumulative feed intake was recorded per pen, and the average daily feed intake (**ADFI**) and the feed to gain ratio (**F/G**) at 1-21 d, 22-53 d, and 1-53 d were calculated accordingly.

Fecal excretion analysis

The collected fresh feces were dried in the oven at 65°C for 72 h, and put indoors at room temperature for 24 h to regain moisture, then ground to powder and passed through a 0.425-mm sieve to prepare dry fecal samples for further determination. The contents of Fe, Cu, Zn, and Mn in fecal samples were measured according to the methods of National Standards of the People's Republic of GB 5009.268-2016. Fecal samples (1g) were weighed and mixed acid of concentrated nitric acid and perchloric acid (4:1) of 12 mL was added to soak in a conical bottle overnight, and then slowly heated to 200-230°C in a constant temperature electric heating plate for 1.5-2 h. When the solution was clarified, distilled water (Watsons, China) was added to remove the residual perchloric acid followed by adding the remaining 2 mL of the solution to stop digestion. After cooling, it was transferred to a 50 mL flask without loss. After constant volume, the insoluble impurities were filtered, and the supernatants were used for determinations. The fecal contents of Zn, Fe, Cu and Mn were analyzed by flame atomic absorption spectrophotometer (AA-7000, Shimadzu, Japan) with wavelengths of 213.9 nm, 248.3 nm, 279.5 nm and 324.8 nm, respectively.

RNA extraction, cDNA synthesis, and qPCR analysis for gene expression

The total DNA was extracted from the jejunal mucosal samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The experimental procedure of RNA extraction and cDNA synthesis was performed according to the previous methods described by Hemida et al. (2023). The purity and concentration of RNA were determined using a DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA). The total RNA (1 μg) was used to synthesize cDNA using a reverse transcription kit (Takara, Japan). Fluorescence qPCR was subsequently performed with iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad, USA) in a QuantStudio 3 Flex real-time system (Applied Biosystems Instruments, Thermo Fisher Scientific, San Jose, CA, USA). The reaction mixture has a total volume of 10 μL, which was consisted of 5 μL of iTaq^{TM} Universal SYBR Green Supermix, 0.5 μM of forward and reverse primers, 2 μL of cDNA, and 2 μL ddH_2O. The fluorescence qPCR reaction was 95 °C for 30 s followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s). Primers (Table 1) used in this study were designed using Primer Premier 5.0 software (Applied Biosystems, USA) and then synthesized by Ige Biotech Co. (Guangzhou, China). The internal reference gene β-actin was used as an internal control with three replicates for each sample, and the fold change of the target gene was calculated for each sample using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1
Primer sequences for quantitative real-time PCR.

Genes[a]	Primer sequences[b]	GenBank access number
Occludin	F-ACGGCAGCACCTACCTCAA R-GGGCGAAGAAGCAGATGAG	XM 025144247.2
Claudin-1	F-CATACTCCTGGGTCTGGTTGGT R-GACAGCCATCCGCATCTTCT	NM 001013611.2
Claudin-5	F-CATCACTTCTCCTTCGTCAGC R-GCACAAAGATCTCCCAGGTC	NM_204201
ZO-1	F-CTTCAGGTGTTTCTCTTCCTCCTC R-CTGTGGTTTCATGGCTGGATC	XM_413773
TNF-α	F-GAGCGTTGACTTGGCTGTC R-AAGCAACAACCAGCTATGCAC	GU230788.1
TGF-β	F-CGGGACGGATGAGAAGAA R-TCGGCGCTCCAGATGTAC	NT_176262.1
IL-1β	F-ACTGGGCATCAAGGGCTACA R-GCTGTCCAGGCGGTAGAAGA	Y15006.1
IL-6	F-CTCCTCGCCAATCTGAAGTC R-CCTCACGGTCTTCTCCATAAAC	NM_204628
IL-8	F-GGCTTGCTAGGGGAAATGA R-AGCTGACTCTGACTAGGAAACTGT	DQ393272.2
SIgA	F-GTCACCGTCACCTGGACTACA R-ACCGATGGTCTCCTTCACATC	S40610
Bax	F-ATCGTCGCCTTCTTCGAGTT R-ATCCCATCCTCCGTTGTCCT	XM_204725
Bcl-2	F-TCGCGCCGCTACCAGAGGGACTTC R-CCGGTTGACGCTCTCGACGCACAT	NM_205339
Caspase-3	F-GGCTCCTGGTTTATTCAGTCTC R-ATTCTGCCACTCTGCGATTT	NM_204725.1
SOD	F-AGGGGGTCATCCACTTCC R-CCCATTTGTGTTGTCTCCAA	NM_205064.1
SOD2	F-CTGACCTGCCTTACGACTATG R-CGCCTCTTTGTATTTCTCCTCT	NM_204211.1
CAT	F-TGAGTCTTCGCCCTGAGTCT R-TAACAGCTCCCCCACTAGCA	NM_001031215.2
GPX4	F-AGAATGTGCGCTCAGGCG R-ACCGCGGTCTTTCCTCATTT	NM_204220.3
β-actin	F-ATGATATTGCTGCGCTCGTT R-TCTTTCTGGCCCATACCAACC	AY550069

[a] ZO-1, zonula occludens-1; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor-β; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; SIgA, secretory immunoglobulin A; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; SOD, superoxide dismutase; SOD2, superoxide dismutase 2; CAT, catalase; GPX4, glutathione peroxidase 4.
[b] F forward; R, reverse.

16 S rRNA sequencing for gut microbiota composition and function

The microbial genomic DNA in cecal contents was extracted by using and Stool DNA Kit (TianGen, China). The V3 to V4 regions of the 16 S rRNA gene was sequenced using universal primers (515F: 5′-CCTAYGGGRBGCASCAG-3′; 816R: 5′-GGACTACNNGGGTATCTAAT-3′). Quantified libraries were pooled and sequenced on Illumina HiSeq 2500 PE250 platform (Novogene Bioinformatics Technology Co., Ltd, Tianjin, China), according to effective library concentration and data amount required. Data split, sequence assembly, data filtration, and chimera removal were performed on sequencing samples to obtain effective tags. For the effective tags obtained, denoise was performed with DADA2 in the QIIME2 software (Version QIIME2-202006) to obtain initial amplicon sequence variants (ASVs). The relative abundances of top 10 most abundant bacteria at the phylum, family, order, and genus levels were calculated using the taxa plugin. The results of α-diversity included Shannon index, Simpson index, Chao1, and Goods_coverage were provided. Moreover, the β-diversity index and unweighted pair-group method with arithmetic means (UPGMA) clustering were used to evaluate treatment differences in complexity of species diversity. The differences in the relative abundances of microbiota among treatments were further analyzed by linear discriminant analysis effect size (LEfSe), t-test analysis, and MetagenomeSeq analysis. The datasets presented in this study can be found in the NCBI online repositories under project PRJNA1138418.

Statistical analysis

Data were analyzed by one-way ANOVA using the IBM SPSS Statistics V26.0 software package (IBM Corp., Armonk, NY, USA). Duncan' multiple comparisons were used to test the significance of the differences between treatment means. The results were presented as the mean and pooled standard error (SEM). Differences were considered significantly expressed at a *P*-value of <0.05 and with a significant tendency at $0.05 \leq P < 0.10$. Spearman's correlation analysis was used to analyze the relationship between host phenotype variables and gut microbiota.

Results

Growth performance

As shown in Table 2, dietary replacement of ITM with OTM at either lower (OTM300) or medium (OTM500) levels significantly increased the ADG during the stages (22-53 d) and the entire period (1-53 d) in Chinese yellow-feathered broilers than those of other groups ($P < 0.05$). However, there were no significant effects of dietary OTM treatments in the ADG, ADFI, and F/G at 1-21 d as well as the ADFI and F/G at 22-53 d and the entire period (1-53d) ($P > 0.05$).

Fecal mineral excretions

As shown in Table 3, compared with PC group, dietary lower (OTM300) or medium (OTM500) levels of OTM could significantly reduce the emissions of Zn, Fe, Cu, and Mn in the feces of Chinese yellow-feathered broilers ($P < 0.05$).

Intestinal morphology

As shown in Table 4, dietary OTM treatments significantly increased the crypt depth in the duodenum of Chinese yellow-feathered broilers compared to the negative control group and ITM300 groups ($P < 0.05$). The ratio of villus height to crypt depth **(V/C)** in the duodenum of OTM group was significantly higher than that in the NC group ($P < 0.05$). Moreover, the ratio of jejunal V/C in OTM300 group was significantly higher than control and ITM groups ($P < 0.05$). In addition, no significant effect of OTM treatment was detected on the crypt depth, villus height, and the ratio of V/C in the ileum ($P > 0.05$).

Intestinal gene expression

As shown in Fig. 1, dietary OTM500 supplementation could significantly upregulate the mRNA expression of ZO-1, claudin-1, CAT and GPX4 in the jejunal mucosa of Chinese yellow-feathered broilers compared to the ITM treatments ($P < 0.05$). Furthermore, dietary replacement of ITM with OTM at lower (OTM300) levels could significantly upregulated the mRNA expression of occludin and claudin-5 in the jejunal mucosa of Chinese yellow-feathered broilers, when compared with NC group and ITM300 group ($P < 0.05$). The mRNA expression of TGF-β and Bcl-2 in the jejunal mucosa was significantly upregulated by dietary OTM treatments ($P < 0.05$). Moreover, dietary OTM supplementation had a trend of downregulating the mRNA expression of IL-1β in the jejunal mucosa of Chinese yellow-feathered broilers ($0.05 \leq P < 0.10$). However, there was no significant difference in IL-6, TNF-α, SIgA, Bax, SOD and SOD2 mRNA expression in the jejunal mucosa of Chinese yellow-feathered broilers among groups ($P > 0.05$).

Cecal microbiota analysis by 16 S rRNA sequencing

As shown in Fig. 2A, at the phylum level, the major phyla were Firmicutes, Bacteroidota, and Proteobacteria. The top 10 family, order and genera and their relative abundances were shown in Fig. 2B-D.

Table 2
Effects of organic trace minerals on growth performance in yellow-Chinese feathered broilers.

Item	NC	PC	ITM300	ITM500	OTM300	OTM500	SEM	*P*-value
BW, g								
d 0	31.19	31.16	31.22	31.20	31.19	31.20	0.01	0.215
d 21	453.45	455.64	459.13	453.97	460.13	466.63	1.91	0.359
d 53	2128.09	2121.12	2108.10	2115.11	2155.37	2157.01	8.77	0.476
1-21 d								
ADFI, g/d	34.11	33.51	34.20	34.39	34.09	34.16	0.15	0.676
ADG, g/d	19.93	20.11	20.51	20.39	20.02	20.38	0.12	0.649
F/G, g/g	1.71	1.67	1.67	1.69	1.71	1.68	0.01	0.264
22-53 d								
ADFI, g/d	140.54	136.17	136.31	140.17	138.88	145.14	1.34	0.426
ADG, g/d	51.37[b]	51.59[b]	51.19[b]	51.47[b]	53.78[a]	54.13[a]	0.32	0.005
F/G, g/g	2.74	2.64	2.66	2.72	2.59	2.68	0.03	0.698
1-53 d								
ADFI, g/d	98.37	95.49	95.85	98.26	97.36	101.17	0.83	0.416
ADG, g/d	38.91[b]	39.11[b]	39.04[b]	39.16[b]	40.40[a]	40.76[a]	0.19	0.004
F/G, g/g	2.53	2.44	2.46	2.51	2.41	2.48	0.02	0.669

[1]Result for mean ± standard error ($n = 8$).
[ab] The means with no common superscripts within each row are significantly different ($P < 0.05$), $0.05 \leq P < 0.10$ indicates a significant trend. Abbreviations: ADG, average daily gain; ADFI, average feed intake; F/G, feed to gain ratio.

Table 3
Effects of organic trace minerals on fecal mineral excretions in Chinese yellow-feathered broilers.

Item	NC	PC	ITM300	ITM500	OTM300	OTM500	SEM	*P*-value
Zn	100.45[c]	175.47[a]	135.58[b]	159.34[a]	125.51[b]	130.64[b]	4.69	<0.001
Fe	635.88[c]	925.65[a]	715.68[bc]	837.74[ab]	692.05[c]	636.79[c]	22.34	0.001
Cu	10.46[b]	17.80[a]	10.95[b]	12.05[b]	11.84[b]	11.34[b]	0.51	<0.001
Mn	203.90[b]	278.20[a]	268.34[a]	286.79[a]	220.85[b]	226.50[b]	7.29	<0.001

[1]Result for mean ± standard error ($n = 8$).
[ab] The means with no common superscripts within each row are significantly different ($P < 0.05$), $0.05 \leq P < 0.10$ indicates a significant trend.

Table 4
Effects of organic trace minerals on intestinal morphology in Chinese yellow-feathered broilers.

Item	NC	PC	ITM300	ITM500	OTM300	OTM500	SEM	*P*-value
Duodenum								
Villus height, μm	1654.71	1730.59	1674.50	1686.94	1515.84	1485.96	26.65	0.061
Crypt depth, μm	162.16 [a]	146.80 [ab]	162.24[a]	148.09 [ab]	132.08 [b]	131.55 [b]	5.92	0.004
V/C	10.15 [b]	11.57 [ab]	10.57 [ab]	12.02 [a]	11.75 [a]	11.79 [a]	0.21	0.045
Jejunum								
Villus height, μm	1557.19	1565.84	1538.28	1524.30	1671.56	1673.96	22.97	0.210
Crypt depth, μm	167.11	154.59	163.77	157.88	148.05	158.53	2.33	0.205
V/C	9.74[b]	10.17 [b]	9.42[b]	9.66 [b]	11.35 [a]	10.53 [ab]	0.14	0.006
Ileum								
Villus height, μm	1242.62	1246.18	1055.39	1250.55	1284.66	1217.92	28.74	0.242
Crypt depth, μm	136.16	155.73	136.43	139.97	148.14	167.41	3.91	0.121
V/C	9.63	8.04	7.85	8.90	8.51	7.36	0.25	0.138

[1]Result for mean ± standard error ($n = 8$).
[ab] The means with no common superscripts within each row are significantly different ($P < 0.05$), $0.05 \leq P < 0.10$ indicates a significant trend. Abbreviations: V/C, the ratio of villus height to crypt depth.

Furthermore, dietary OTM supplementation could significantly decrease the abundance of Bacteroidota (phylum), *Lachnospiraceae* (family), *Lactobacillaceae* (family), and *Lactobacillales* (order), while increasing the abundance of Firmicutes (phylum), and *[Eubacterium]_coprostanoligenes_group* (family) (Fig. 3). However, there were no significant effects of dietary OTM treatments on top 10 genera and their relative abundances in the cecal contents of Chinese yellow-feathered broilers.

The α-diversity of cecal microbiota of Chinese yellow-feathered broilers among the treatments are shown in Fig. 4. The Good-s_coverage indexes were all higher than 99%, indicating that the sequencing results could reliably and accurately reflect the real situation of cecal microbiota of Chinese yellow-feathered broilers. Dietary supplementation with OTM500 significantly decreased the α-diversity parameters including Chao 1 and Observed_features index in the Chinese yellow-feathered broilers ($P < 0.05$). However, there were no significant differences in the Shannon index, Simpson index and Pielou_e index among groups ($P > 0.05$).

The β diversity index, PCoA, and UPGMA were used to evaluate the similarities and differences of gut microbiota among groups in Chinese yellow-feathered broilers. As shown in Fig. 5A, OTM500 group significantly increase the β diversity index compared with ITM treatments, while the β diversity index in ITM500 group was significantly decreased ($P < 0.05$). Moreover, the UPGMA analysis (Fig. 5B) based on unweighted uniFrac distance revealed distinct differences of cecal microbiota fed different sources of trace elements. The LEfSe analysis (Fig. 5C) also showed that 10 discriminative species were identified among six groups. In particular, *Lactobacillales* and *Lachnospirales* (genus), as well as *Lactobacillaceae, Lachnospiraceae* and *Rikenellaceae* at family level were enriched in NC group while PC group enriched *Rikenellaceae* (family) and *Alistipes* (genus). Furthermore, *Ralstonia* (genus),

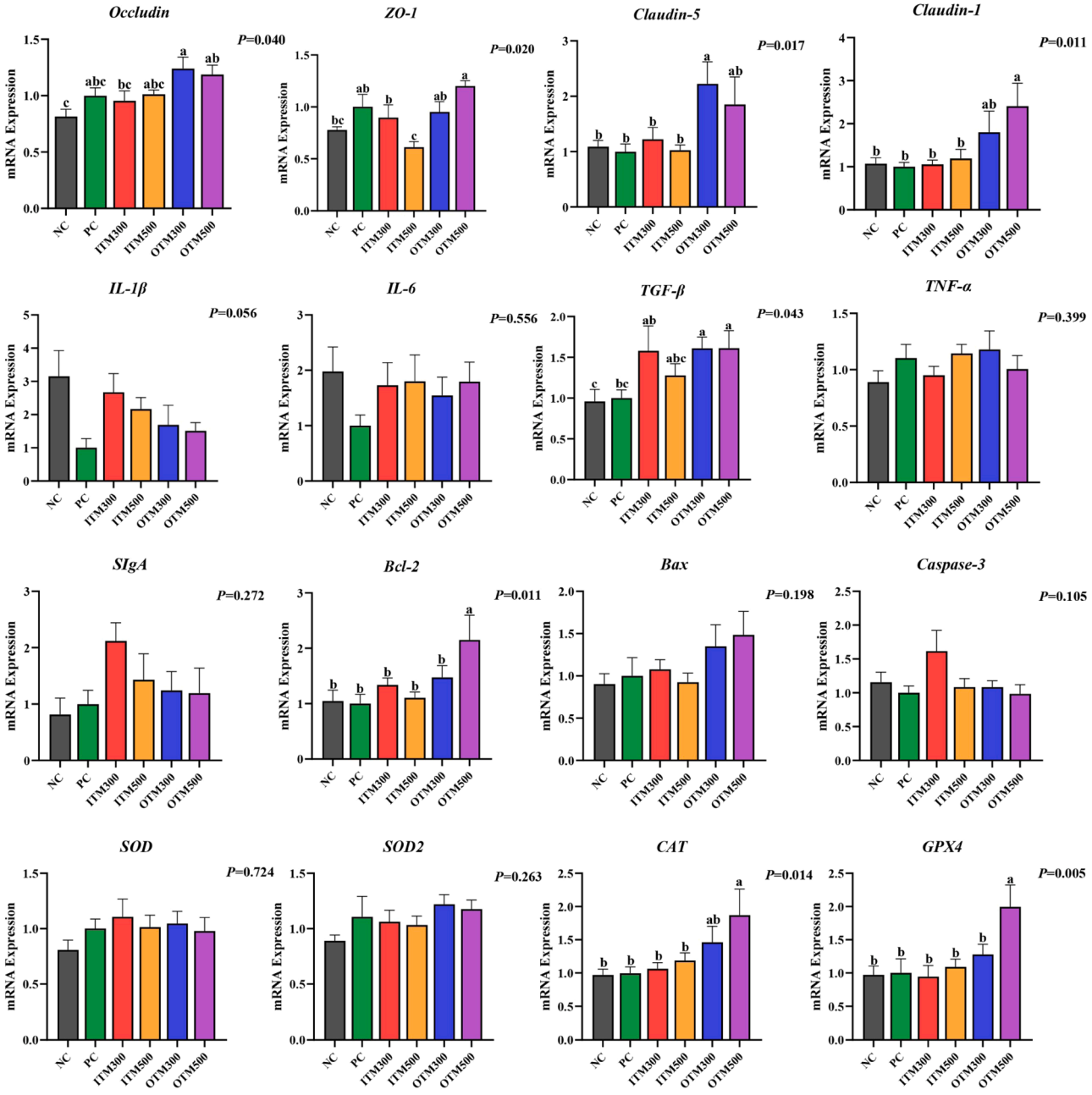

Fig. 1. Effects of dietary compound organic trace minerals on mRNA expression of intestinal tight junction proteins and inflammatory factors in Chinese yellow-feathered broilers.

[a,b] indicate a significant difference at $P < 0.05$ ($n = 8$). Abbreviations: ZO-1, zonula occludens-1; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor-β; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; SIgA, secretory immunoglobulin A; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; SOD, superoxide dismutase; SOD2, superoxide dismutase 2; CAT, catalase; GPX4, glutathione peroxidase 4; NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

Burkholderiaceae (family), and *Eubacterium_coprostanoligenes_group* (family) were enriched in OTM500 group.

Further MetagenomeSeq analysis and T-test compared differences in taxonomic abundances between treatments. As shown in Fig. 5D, MetagenomeSeq analysis showed that the relative abundances of *Ralstonia* and *Rikenellaceae_RC9_gut_group* were significantly reduced in ITM300 and OTM300 groups, regardless of inorganic or organic sources ($P <$ 0.05). The T-test analysis (Fig. 6) showed that compared with OTM500 group, the relative abundance of *[Eubacterium]_hallii_group* was significantly increased in PC, ITM500 and OTM300 groups. Moreover, ITM300 group significantly increased the relative abundance of *Christensenellaceae_R-7_group, Alistipes* and CHKCI001 that compared with ITM500 group.

As shown in Fig. 7, the third level of functional prediction of the heatmap showed that OTM300 treatment significantly enriched the microbial metabolic pathways such as carbon fixation, amino acid related enzymes, mitochondrial biosynthesis, and DNA replication while OTM500 treatment significantly enriched the microbial metabolic pathways including ABC transporters, pyruvate metabolism, glycogen decomposition and synthesis, DNA replication, as well as cysteine and

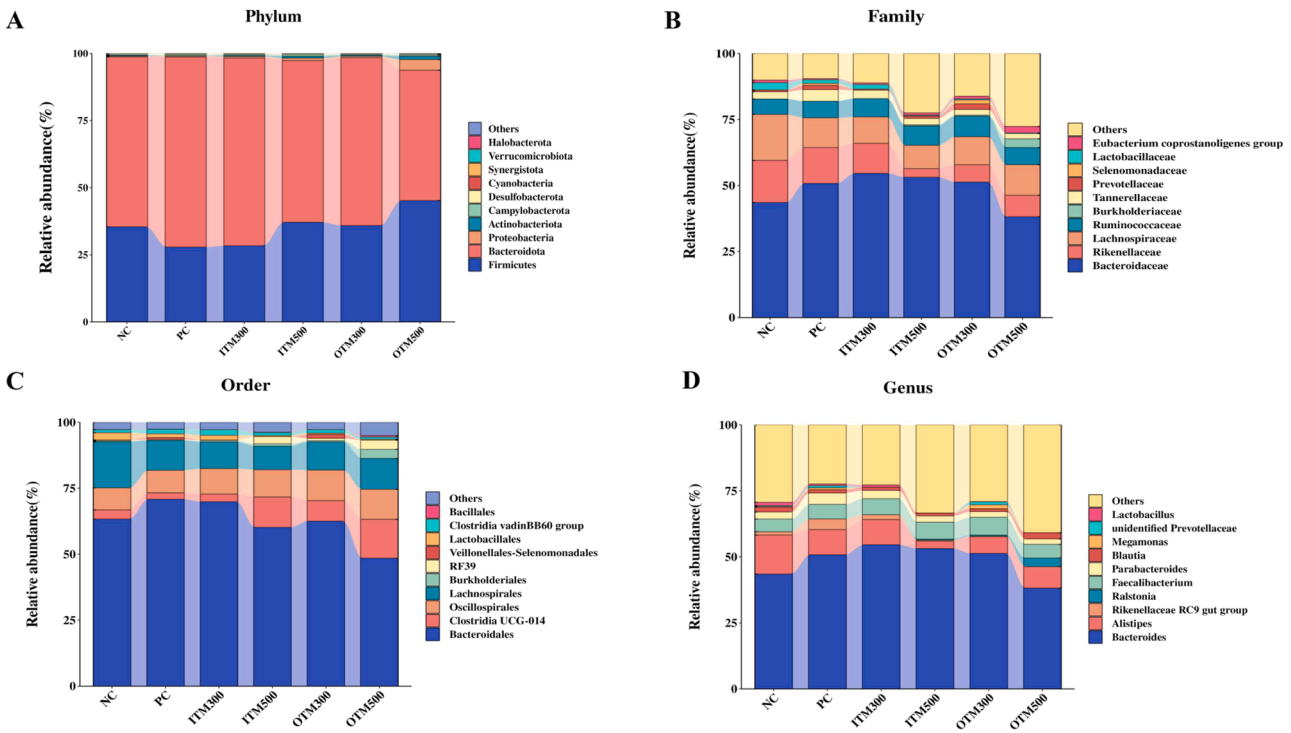

Fig. 2. Effect of dietary organic trace minerals on the composition of gut microbiota in the cecum of Chinese yellow-feathered broilers. The top 10 microbial community at the phylum (A), class (B), order (C), and genus (D) levels.
[a,b] indicate a significant difference at $P < 0.05$ ($n = 8$). Abbreviations: NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

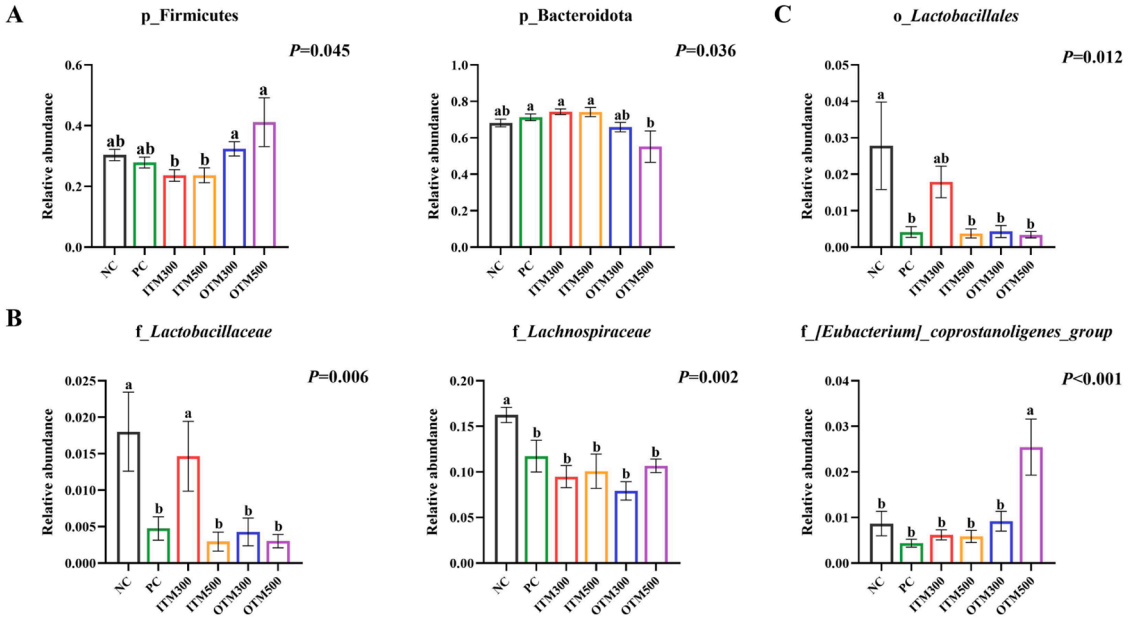

Fig. 3. Effect of dietary organic trace minerals on the differential level of gut microbiota in the cecum of Chinese yellow-feathered broilers. The differential bacteria at the phylum (A), family (B), and order (C) levels among treatments. Differences were determined by one-way ANOVA followed by Duncan's multiple comparison.
[a,b] indicate a significant difference at $P < 0.05$ ($n = 8$). Abbreviations: NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

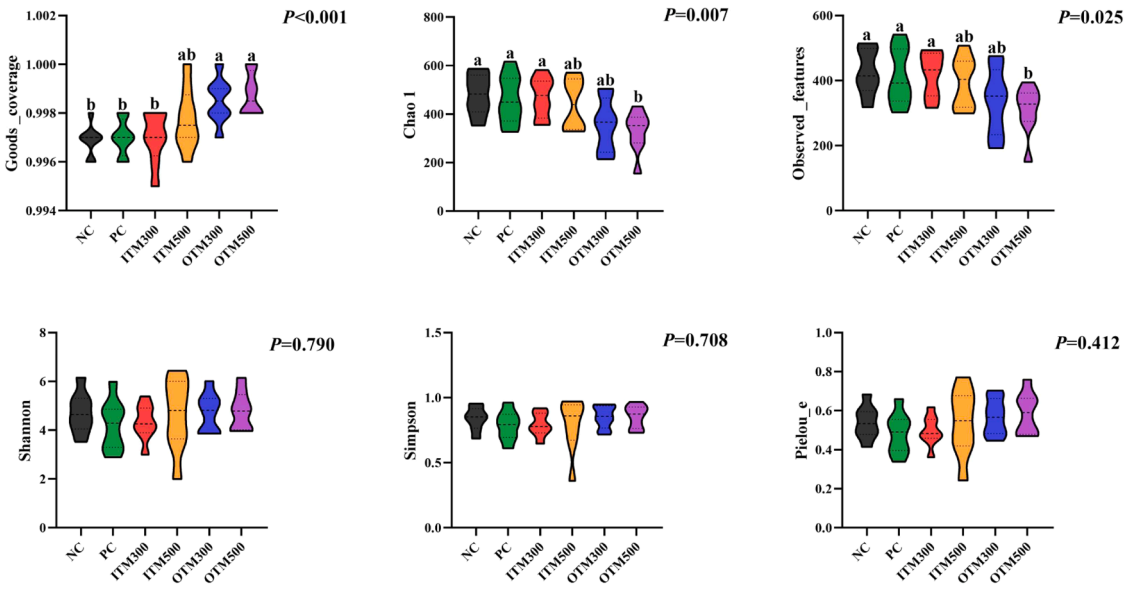

Fig. 4. Effect of dietary organic trace minerals on the microbial diversity of cecum in Chinese yellow-feathered broilers.
[a,b] indicate a significant difference at $P < 0.05$ ($n = 8$). Abbreviations: NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

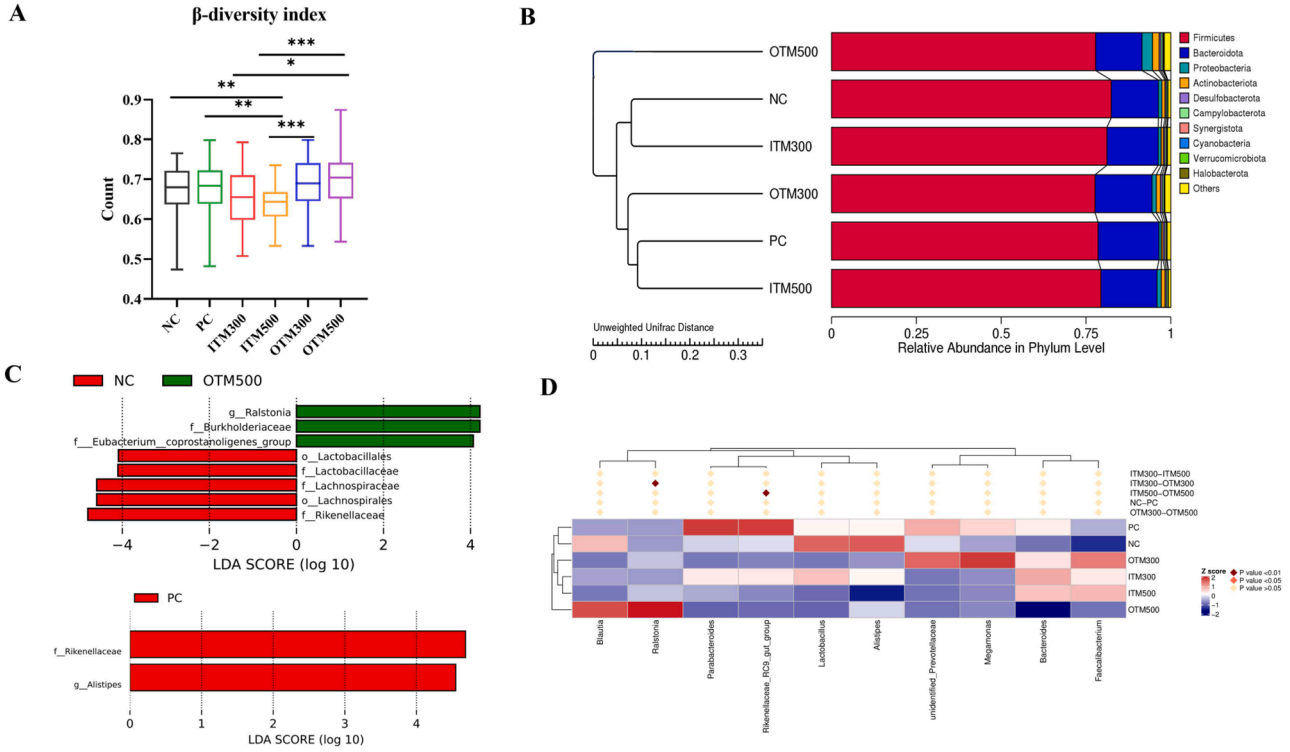

Fig. 5. The β-diversity index and the MetagenomeSeq analysis of cecal microbiota in Chinese yellow-feathered broilers by dietary organic trace minerals supplementation. (A) β-diversity index of cecal microbiota. (B) UPGMA analysis based on unweighted UniFrac distance. (C) The LEfSe analysis (LDA score ≥4). (D) The heat map of MetagenomeSeq analysis at the genus level.
Abbreviations: NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

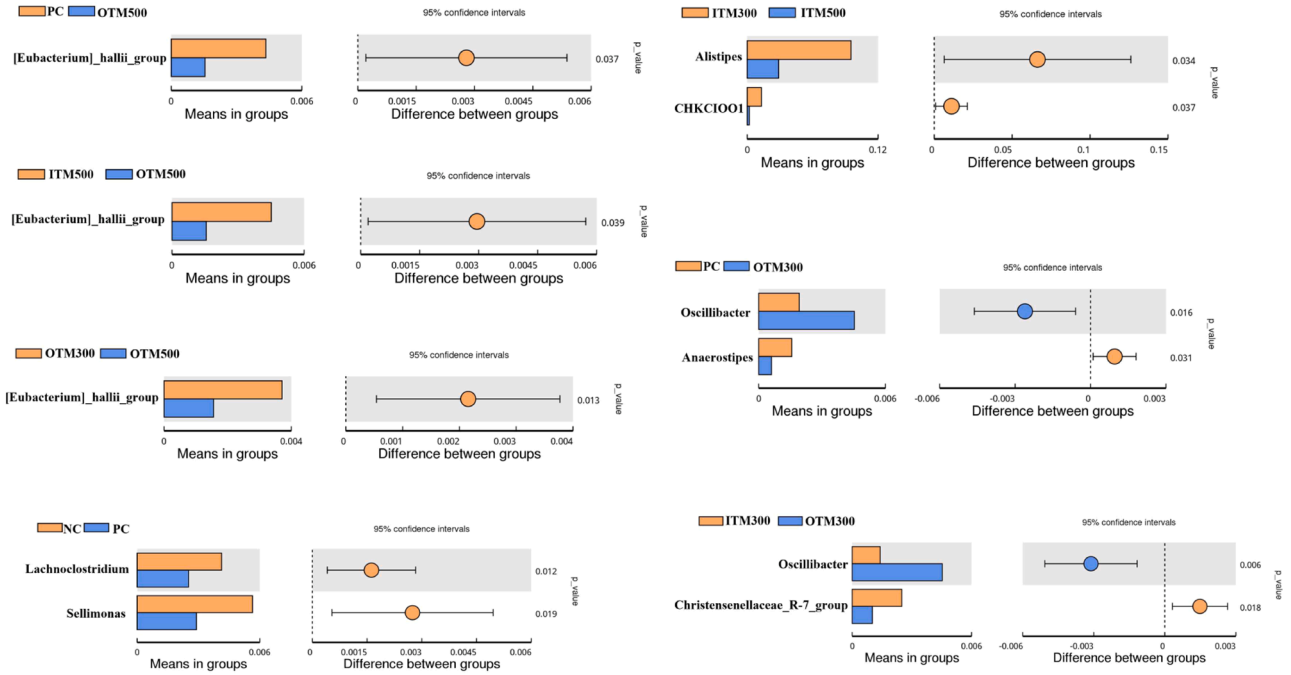

Fig. 6. T-test analysis for the significant changes of differential cecal microbiota at genus levels in Chinese yellow-feathered broilers. Abbreviations: NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

methionine metabolism.

Spearman correlation with cecal microbiota and host phenotype parameters

Further spearman correlation analysis (Fig. 8) showed that there was significantly correlation between differential cecal microbiota with intestinal morphology, intestinal gene expression and fecal mineral excretion in Chinese yellow-feathered broilers. Specifically, the V/C ratio in the jejunum and the mRNA expression of occludin were positively associated with the abundances of Firmicutes (phylum), but were negatively associated with the abundances of Bacteroidota (phylum) and *Lactobacillales* (order). Additionally, the abundances of *[Eubacterium]_hallii_group* (genus), and *Anaerostipes* (genus) were positively associated with crypt depth in the duodenum ($P < 0.05$). Furthermore, the abundances of *[Eubacterium]_coprostanoligenes_group (genus)* were positively associated with the mRNA expression of claudin-1, TGF-β and Bcl-2, as well as the abundances of *Burkholderiaceae (family) and Ralstonia (genus)* were positively associated with the mRNA expression of TGF-β and Bcl-2 ($P < 0.05$). Moreover, the abundances of *Lachnoclostridium* (genus) were negatively associated with the fecal concentrations of Fe, Mn, and Zn, but were positively associated with the ADG at 22-53 d and entire period (1-53 d) ($P < 0.05$).

Discussion

For decades, dietary supplementation with inorganic trace minerals (**ITM**) is a common and cost-effective way of optimizing animal health and production, but the less effective bioavailability of ITM would increase the risk of environmental burdens (Franklin et al., 2022). Instead, the organic trace minerals (**OTM**) in the forms of proteinates and amino acid chelates represent an alternative environmentally sustainable way to reduce mineral excretion by increasing the absorption of trace elements with higher bioavailability than ITM in the body (Zhang et al., 2017; Torres and Korver, 2018). Studies have confirmed that low doses of chelating OTM could be alternative sources of ITM to keep normal growth and antioxidant status of animals, and supplementing the same level of chelating OTM could entirely replace ITM to improve the animal production performance (Ghasemi et al., 2020). The trace mineral sources (organic vs. inorganic) had been shown to induce no significant differences in feed intake, body weight gain, feed conversion ratio or livability of Ross 308 broilers (M'Sadeq et al., 2018). Other researchers also showed that replacing inorganic minerals with low doses of OTM did not negatively affect growth performance in 817 white-feathered broilers (Kong et al., 2022) and Cobb 400 broilers (Savaram Venkata et al., 2021). However, the present study found that equivalent replacement of ITMs with low or medium levels of amino acids-chelated compound OTM (OTM300 and OTM500) significantly increased the ADG of Chinese yellow-feathered broilers during 22-53 d and 1-53 d. Consistently, previous research found that low levels of OTM could improve body weight and livability and reduce the feed conversion ratio in Ross 708 broiler chicks (Vieira et al., 2020). Similarly, previous studies have found that dietary supplementation with Fe or Zn glycine chelate in place of inorganic sources of Fe or Zn both improved growth performance of broilers (Ma et al., 2012; Zhang et al., 2017). Interestingly, dietary OTM was found to improve performance of slow broiler breeders and their offspring (Araújo et al., 2019; Güz et al., 2022). The discrepancy of varied results from different studies might be related to various factors, including the genetic background, broiler ages, feeding management, mineral supplementation levels, and dietary composition and nutrient levels.

When dietary mineral supplementation exceeds the maximum requirement of animals to maintain normal growth, excess minerals are largely excreted with feces, which would cause environmental pollution in animal production. Increasing evidence has shown that dietary low doses of OTM supplementation not only had no negative effect on animal performance, but also significantly reduced trace element emissions in feces (Marco et al., 2017; Zhu et al., 2019). Notably, amino acid-chelated OTM represent an effective way to reduce fecal mineral excretion to the environment due to their higher bioavailability (Singh et al., 2015; Qiu et al., 2020). Consistently, we found that dietary supplementation with medium and low levels of amino acid-chelated

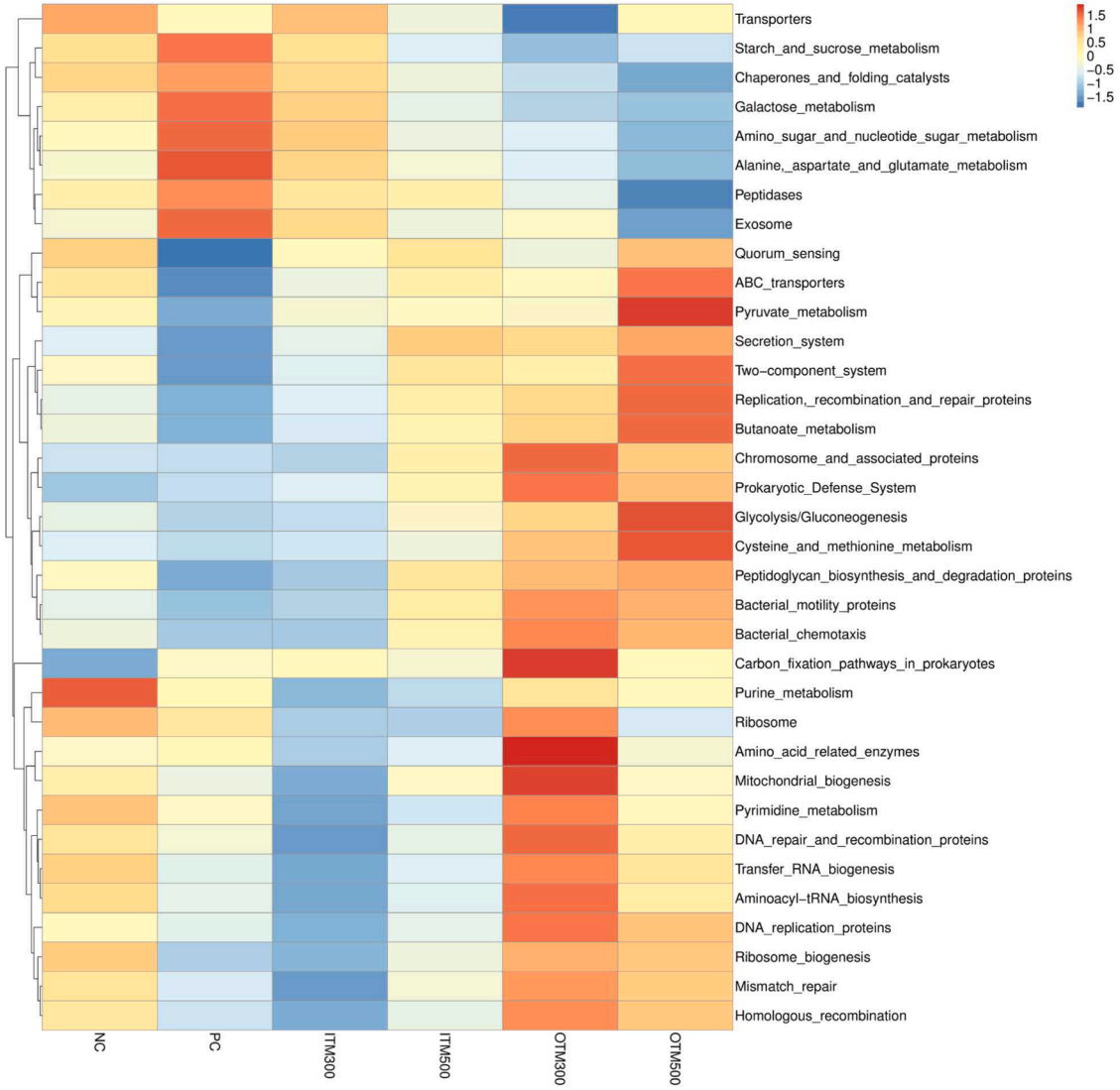

Fig. 7. Predicted microbial function of cecal microbiota in Chinese yellow-feathered broilers treated with dietary organic trace minerals. Abbreviations: NC, negative control fed diet without inorganic trace mineral (ITM) or organic trace mineral (OTM) premix; PC, positive control fed diet supplemented with ITM premix at 1,000 mg/kg; ITM300, control diet supplemented with ITM premix at 300 mg/kg; ITM500, control diet supplemented with ITM premix at 500 mg/kg; OTM300, control diet supplemented with OTM premix at 300 mg/kg; OTM500, control diet supplemented with OTM premix at 500 mg/kg.

compound OTM could significantly reduce the excretion of Zn, Fe, Cu and Mn in feces of Chinese yellow-feathered broilers in present study. Similarly, a previous study on chicks found that replacement of ITM in diets with OTM reduced fecal excretion of Zn, Cu, Mn and reduced mineral excretion in bedding (Vieira et al., 2020). Furthermore, lower levels of 30% to 50% of compound OTM (Cu, Zn, Mg, Fe) considerably reduced the fecal mineral contents in aged laying hens compared with those in 100% ITM (Zhang et al., 2021). Moreover, dietary supplementation of 50% NRC recommended levels of OTM promoted optimal egg production performance, mineral deposition, and reduced mineral excretion in laying hens (Yang et al., 2021). Meanwhile, replacing 100% ITMs with OTM significantly reduced the contents of Cu, Zn, and Mg in feces in growing-finishing pigs (Xiong et al., 2023). These results and our data confirmed that lower levels of OTM supplementation could achieve higher bioavailability of trace elements and reduce mineral excretion of animals, thus helping protect the environment. However, the basal diet contains certain amount of trace elements, and its anti-nutrient factors and the unavailable form may greatly reduce the absorption and utilization of trace elements (Byrne and Murphy, 2022). The accurate bioavailability of diets among treatments containing OTM or ITM is still unclear and needs to be further explored.

Trace elements are essential nutrients for maintaining the intestinal morphology and function of animals. The small intestine is the main site of the absorption of trace elements. In the current study, we found that adding 300 mg/kg compound OTM significantly reduced the duodenal crypt depth and improved the V/C ratio in the jejunum. This might indicate that OTM could improve intestinal morphology for higher mineral absorption and utilization in Chinese yellow-feathered broilers, which may be related to the improved bioavailability of chelating complex OTM. Our results were consistent with previous research showing that compound amino acid sources of Zn, Cu, and Mn at lower levels improved duodenal villus height and the ratio of V/C in the jejunum of laying hens (Santos et al., 2024). Furthermore, previous studies have found that dietary OTM could improve the intestinal morphology of broilers or laying hens compared with ITM (Echeverry et al., 2016; Cao et al., 2023).

Tight junctions were regarded as the effectors of mucosal homeostasis, which played a key role in maintaining the integrity of the intestinal barrier (Suzuki, 2020; Zuo et al., 2020). Previous studies have found that low or equivalent levels of OTM replacing ITM could

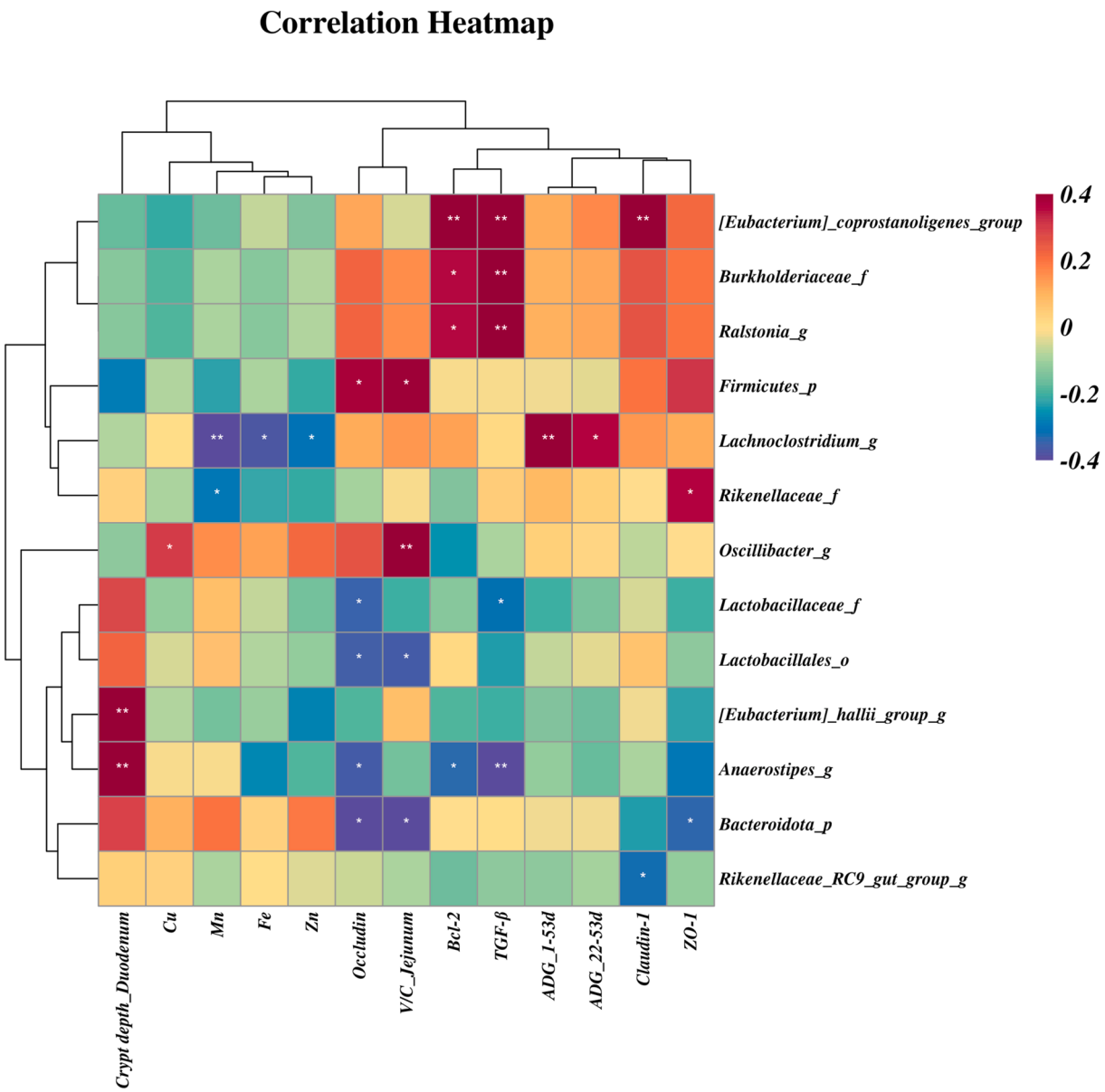

Fig. 8. Spearman correlation between cecal microbiota and host phenotype parameters in Chinese yellow-feathered broilers. Abbreviations: ADG, average daily gain; ADFI, average feed intake; V/C, the ratio of villus height to crypt depth; Bcl-2, B-cell lymphoma-2; TGF- β, transforming growth factor-β.

significantly increase the occludin mRNA expression level in the duodenum of broilers (Wang et al., 2023a). Similarly, the current study showed that dietary supplementation with the OTM enhanced intestinal mucosal barrier in the jejunum of Chinese yellow-feathered broilers as reflected by the upregulated mRNA of claudin-1 and ZO-1 in OTM500 group, and occludin and claudin-5 in OTM300 group. This was in accordance with a recent study that dietary medium concentration of OTM could significantly increase ileum ZO-1 and occludin protein levels in weaned piglets (Wang et al., 2023b). Additionally, our recent results have confirmed that dietary supplementation with low or medium OTM could enhance the antioxidant capacity of plasma and liver in Chinese yellow-feathered broilers (Nie et al., 2024). Here, our current study also found that dietary supplementation with medium OTM could significantly increase the CAT and GPX4 expression in the jejunal mucosal of Chinese yellow feathered broilers. Thus, dietary supplementation of OTM might promote intestinal expression of tight junction proteins and antioxidant gene expression to improve intestinal barrier functions and antioxidant capacity in Chinese yellow-feathered broilers.

Increasing evidence has shown that OTM are involved in the regulation of immune function by increasing the concentration of immunoglobulin and reducing the expression of proinflammatory cytokines, thereby improving the immune defense system of animals (Manangi et al., 2015; Jarosz et al., 2017b). Therefore, to further explore the effects of OTM on intestinal health, we examined the expression of cytokine genes related to the development of inflammation. We found that dietary OTM increased the mRNA expression of TGF-β and Bcl-2 in the jejunum mucosa, and had a tendency to decrease IL-1β mRNA expression in the jejunum mucosa of Chinese yellow-feathered broilers. Our results were in agreement with the previous study that supplementation with OTM maintained intestinal homeostasis by inhibiting the expression of proinflammatory factors and inflammatory immune responses in the jejunum and ileum of broilers (Jarosz et al., 2017a). Furthermore, previous study found that organic Zn supplementation improved the immune defense function of broilers challenged with *Eimeria tenella* (Bun et al., 2011).

Importantly, intestinal homeostasis plays a central role in the regulation of mineral bioavailability in animals, and the interaction between trace elements and intestinal microbiota would further impact intestinal

health (Barra et al., 2021; Pajarillo et al., 2021). Recent studies have shown that dietary organic Se supplementation can enrich intestinal microbiota and promote intestinal health of laying hens (Li et al., 2024). In addition, dietary supplementation with Zn amino acid complexes resulted in a decreased abundance of several genera belonging to the phylum Proteobacteria in the ileum of broilers (De Grande et al., 2020). Another study showed that dietary supplementation with OTM increased Shanno index of cecal microbiota in broilers (Wang et al., 2023a). However, the effect of the compound amino acid-chelated OTM on the cecal microbiota and its correlation with mineral excretion and gut health of Chinese yellow-feathered broilers remained unclear. Here, we showed that dietary supplementation with compound OTM500 decreased the Chao 1 index, but increased β diversity index in cecal microbiota of Chinese yellow-feathered broilers. Furthermore, the current study found that dietary OTM supplementation could significantly affect the composition and structure of the cecal microbial community, with significant increase of Firmicutes and decrease of Bacteroidetes when compared to ITM, which were related to the enhanced ratio of jejunal V/C and occludin gene expression. Previous studies have supported our results that Firmicutes has a positive effect in the intestinal barrier function, thereby promoting the intestinal health (Henn et al., 2021). Further Lefse and T-test analysis showed that compound OTM significantly upregulated the abundance of *Burkholderiaceae* (family), *Eubacterium_coprostanoligenes_group* (family), and *Oscillibacter* (genus), while downregulating the abundance of *Rikenellaceae RC9 group* (genus), *[Eubacterium]_hallii_group* (genus), and *Lactobacilli* at the family and order levels. Notably, *Lactobacillus species* could sense intestinal Fe levels, and dietary low Fe levels might promote the abundance of *Lactobacillus* (Hu et al., 2019; Das et al., 2020). Similarly, previous study has shown that dietary Zn proteinate supplementation could decrease the abundance of *Lactobacillus* in the ileum of broilers after coccidia and *Clostridium perfringens* challenges (Bortoluzzi et al., 2019). Previous studies have also found that the increased abundance of the *Rikenellaceae RC9 group* may associated with stimulate intestinal inflammation and intestinal barrier damage (Sun et al., 2019), while *Eubacterium_coprostanoligenes_group* could enhance mucosal barrier function and effectively alleviate the development of intestinal mucosal inflammation (Bai et al., 2024). Consistently, our current findings also demonstrated the mRNA expression of claudin-1 was positively associated with the abundance of *Eubacterium_coprostanoligenes_group* (family), but was negatively associated with the abundance of *Rikenellaceae RC9 group* (genus). Further PICRUST prediction of microbial function showed that OTM enriched the microbial metabolic pathways of amino acid metabolism, DNA replication, glycogen decomposition and synthesis, mitochondria, biosynthesis and transporter pathways in Chinese yellow-feathered broilers. Collectively, dietary supplementation of compound OTM might improve the intestinal health and enhance the bioavailability of trace elements of Chinese yellow-feathered broilers by regulating the composition and function of gut microbiota and enhancing the intestinal barrier function.

Conclusion

In conclusion, the current study showed that dietary supplementation of lower doses of amino acids-chelated compound OTM (Fe, Cu, Mn, and Zn) improved growth performance, enhanced intestinal morphology, antioxidant capacity and barrier function, and effectively reduced fecal mineral excretions in Chinese yellow-feathered broilers, thereby helping reduce environmental contamination by trace minerals. Furthermore, the improvement of gut health by dietary amino acids-chelated compound OTM might be related to the composition and function of cecal microbiota in Chinese yellow-feathered broilers. Our current findings might provide new insights into the interplay between intestinal microbiota and dietary trace elements especially in amino acids-chelated organic forms. However, further research is warranted to elucidate the underlying mechanisms between OTM and intestinal microbiota, as well as the utilizable levels of trace minerals in the diets, thereby providing the scientific insights into future application of trace minerals additives in broiler production.

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, 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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