



Original Research Article

Dietary methionine sources and levels modulate the intestinal health status of broiler chickens



Lu Gong ^a, Tahir Mahmood ^b, Yves Mercier ^b, Huiping Xu ^a, Xiaodan Zhang ^a, Yizhu Zhao ^a, Yimeng Luo ^a, Yuming Guo ^{a,*}

^a State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China

^b Adisseo France S.A.S, 03600, Commentry, France

ARTICLE INFO

Article history:

Received 28 January 2023

Received in revised form

28 June 2023

Accepted 7 July 2023

Available online 1 August 2023

Keywords:

Chicken

Methionine

2-Hydroxy-4-(methylthio)-butanoic acid

Intestinal health

Intestinal barrier

ABSTRACT

Given the key role of methionine in biological processes, adequate methionine should be provided to meet the nutritional requirements. DL-2-hydroxy-4-(methylthio)-butanoic acid (DL-HMTBA) has been considered as an important source of methionine. However, the effects of different sources and levels of methionine on the intestinal health status have not been clarified yet. An experiment was carried out to investigate the effects of different dietary sources and levels of methionine on the intestinal epithelial barrier, inflammatory cytokines expression, ileal morphology, microbiota composition, and cecal short chain fatty acids (SCFA) profiles. For this purpose, 720 male Arbor Acre broiler chicks at 1 d old were randomly assigned to a 2 × 3 factorial arrangement with 2 methionine sources (DL-methionine and DL-HMTBA) and 3 total sulfur amino acids (TSAA) levels (80%, 100%, and 120% of Arbor Acre recommendation). The results showed that DL-HMTBA supplementation promoted intestinal physical barrier at both gene expression level of claudin-1 and serum diamine oxidase level ($P < 0.05$), and the inflammatory cytokine *IL-6* mRNA expression was down-regulated by dietary DL-HMTBA supplementation compared with the DL-methionine group ($P < 0.05$). Meanwhile, an upregulated gene expression of claudin-1 and zonula occluden-1 (*ZO-1*) were observed in the low-TSAA treatment on d 14 ($P < 0.05$), whereas this treatment increased the expression of *IL-1 β* and *IL-6* ($P < 0.05$). Villus height to crypt depth ratio was high ($P < 0.05$) in the middle-level TSAA group. Furthermore, DL-HMTBA supplementation optimized the microbiota of the ileum especially the relative abundance of *Lactobacillus*, where the digestion and absorption were completed, and elevated the concentrations of SCFA (acetate, propionate, and butyrate) in the cecal content on d 21 ($P < 0.01$). In conclusion, dietary DL-HMTBA supplementation improved the intestinal barrier function, immune homeostasis and optimized the microbiota to promote intestinal health status in broiler chickens.

© 2023 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

To meet the nutritional requirements of broilers for optimum growth, methionine must be supplemented in diets from synthetic sources, such as DL-methionine or DL-2-hydroxy-4-(methylthio)-

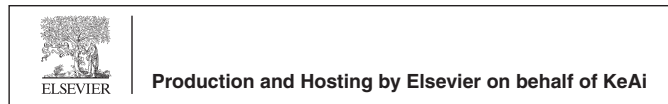
butanoic acid (DL-HMTBA). It is widely recognized that both methionine sources are equally effective in satisfying the growth needs of poultry (Agostini et al., 2016; Vazquez-Anon et al., 2006). According to the metabolic conversion of methionine into cysteine and downstream sulfur components, the practically total sulfur amino acids (TSAA) requirement is fulfilled by supplying only crystalline methionine source in poultry feed formula (Stipanuk, 2004).

Intestinal health is a multifaceted term and comprises many elements, for instance, intestinal permeability (IP), intestinal immune system, intestinal morphology, and the gut microbiome (Khoshbin and Camilleri, 2020; Pelaseyed et al., 2014; Singh et al., 2017; Xu et al., 2003). Thus, optimal health and functioning of the intestine is a prerequisite for improved performance and general

* Corresponding author.

E-mail address: guoyum@cau.edu.cn (Y. Guo).

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.



Production and Hosting by Elsevier on behalf of KeAi

well-being of poultry. In recent years, a variety of nutrients have been recognized to regulate and maintain intestinal health which might depend upon regulating the growth and healing of intestinal mucosa, maintaining the homeostasis of microbiota, and regulating the intestinal immune state (Bauchart-Thevret et al., 2009; Gan et al., 2020; Zhang et al., 2020). Moreover, the appropriate protein level in the diet is mandatory not only for growth but also for the intestinal health of poultry as amino acids being the basic building blocks of protein exert beneficial effects on regulating intestinal homeostasis, thereby maintaining gut health and preventing intestinal damage (Beaumont and Blachier, 2020; Gottardo et al., 2016).

In this regard, methionine has been recognized to maintain the functional integrity of the intestinal barrier (Chen et al., 2014; Ramalingam et al., 2010), promote renewal and regeneration of intestinal epithelium (Bauchart-Thevret et al., 2009), and stimulate intestinal stem cell proliferation (Saito et al., 2017). Once absorbed by the epithelial cells, a fraction of methionine is used for cell metabolism in intestinal cells while the remaining is exported out to the liver via the portal vein and delivered into the blood for utilization of other cells (Garcia and Stipanuk, 1992; Mastrototaro et al., 2016). Consequently, the absorption of methionine in the intestine has a direct or indirect impact on the intestinal health of animals. Small intestine compartments, mainly jejunum and ileum, are the major sites for nutrient absorption in poultry. Furthermore, the intestine constitutes an important part of the immune response and endocrine activity (Martin et al., 2018). Also, optimum gut pH is pivotal for intestinal functionality and nutrient absorption (Nguyen et al., 2020). Notwithstanding a general inconsistency in chyme pH among different species (Krutthai et al., 2015; Wu et al., 2020), the study on Arbor Acres broilers indicated that a high level of methionine decreased pH in the crop, jejunum, and ileum compared with the low level of methionine (Wu et al., 2020).

Ostensibly, gut microbiota disorders can hinder the digestion, absorption, and utilization of nutrients resulting in poor performance and economic losses (Abd et al., 2022). Nava et al. (2009) showed that organic acid (formic and propionic acid) blended with DL-HMTBA increased total bacterial and *Lactobacilli* populations in the chicken gut (Nava et al., 2009). In addition, both DL-methionine and DL-HMTBA may reduce intestinal populations of *Clostridium perfringens* in broiler chickens when used in relatively high concentrations, thus reducing the risk of necrotic enteritis (Dahiya et al., 2007). Some studies have also shown that DL-HMTBA could strengthen the tight junctions (TJ) and protect the Caco-2 cells epithelial barrier in the H₂O₂ or TNF- α induced inflammatory model in vitro (Martín-Venegas et al., 2013). However, the literature on intestinal health with different methionine sources and their levels is still limited and not systematic, and the effects of methionine sources on intestinal health status need to be further explored.

Therefore, in this study, we explored the effects of different dietary methionine sources and levels on the intestinal health status of broilers and provided a novel understanding of gut microbiota and short chain fatty acids (SCFA) involvement in methionine sources-regulated intestinal homeostasis.

2. Materials and methods

2.1. Animal ethics statement

The experiment and all animal procedures were approved by the Animal Care and Use Committee of China Agricultural University (Permit Number: AW51112202-1-1) and were in accordance with the guidelines of the Guide for the Care and Use of Agricultural Animals in Research and Teaching. All efforts were made to minimize the suffering of the animals.

2.2. Broiler feeding and management

The study was conducted at the Zhuozhou experimental station of China Agricultural University. A total of 720 Arbor Acre male broiler chicks at 1 d old were assigned randomly to 6 treatments with 10 replicate pens of 12 broilers each and allotted to 2 methionine sources (DL-methionine vs. DL-HMTBA) \times 3 TSAA levels (80%, 100%, and 120% of Arbor Acre TSAA recommendation) factorial arrangement. Both DL-methionine (Rhodimet NP99, 99% DL-methionine, Adisseo France, SAS) and DL-HMTBA (Rhodimet AT88, 88% OH-methionine, Adisseo France, SAS) were supplemented on an equimolar basis in the experimental diets. The birds were fed the experimental diets from 1 to 21 d of age. The first few weeks post-hatch is considered a critical period for the development of intestine and gut microbiota. Therefore, we hypothesized that any effect of methionine sources and levels would be more conspicuous during the early growth period, so we selected this duration for our experiment. The chickens were fed ad libitum and had free access to drinking water. The room temperature was initially set between 33 and 35 °C and then gradually reduced by 2 to 3 °C according to the age of the birds. All the birds were raised in an environmentally controlled house under 24 h continuous light. The chicken house was disinfected following routine requirements. Methionine sources were incorporated at the expense of maifanite powder to keep other ingredients at the same level between treatments. Table 1 presents the composition and nutrient levels of the experimental diets. Crude protein, gross energy, methionine, cystine and HMTBA in diets were determined according to GB/T 6432-2018, ISO 9831:1998, GB/T 18246-2019, GB/T 18246-2019, GB/T 19371.2-2007, respectively.

2.3. Dissection procedure and sample collection

The effects of dietary methionine sources and levels on growth performance of broiler chickens during the experimental period were shown in Supplementary Table S1. At d 14 and d 21, one bird per replicate representative of the average body weight of the replicate was selected from each treatment. The selected broilers were stunned electrically and killed. The blood was collected to harvest serum by centrifugation at $1,636 \times g$ for 10 min at 4 °C and stored at –20 °C for subsequent analysis. The ileum was collected and divided into anterior (proximal to jejunum), middle (approximately 3 cm), and posterior segments. Tissue sections (about 2 cm) from the middle of the individual ileum were cut and placed into 4% (v/v) paraformaldehyde solution for histomorphometry measurement. The other part of the middle ileum segment was collected in an RNA-free centrifuge tube, snap-frozen in liquid nitrogen, and stored at –80 °C for mRNA analysis. Ileal contents of the posterior segment and cecal contents were collected, respectively, rapidly frozen with liquid nitrogen, and stored at –80 °C for the determination of microbiota and SCFA concentrations.

2.4. Serum diamine oxidase (DAO)

All serum samples were thawed and homogenized before analysis. The content of serum DAO was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (ml036981, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

2.5. RNA extraction and quantitative RT-PCR

Total RNA was isolated using a Trizol reagent (TaKaRa Bio Inc., Kyoto, Japan) following the manufacturer's instructions. Total RNA

Table 1
Ingredient and nutrient levels of diets (% as-fed basis).

Item	DL-Methionine (80% TSAA)	DL-Methionine (100% TSAA)	DL-Methionine (120% TSAA)	DL-HMTBA (80% TSAA)	DL-HMTBA (100% TSAA)	DL-HMTBA (120% TSAA)
Ingredients						
Corn	51.10	51.10	51.10	51.10	51.10	51.10
Soybean meal	35.20	35.20	35.20	35.20	35.20	35.20
Soybean oil	4.10	4.10	4.10	4.10	4.10	4.10
Corn gluten meal	3.20	3.20	3.20	3.20	3.20	3.20
Dicalcium phosphate	2.00	2.00	2.00	2.00	2.00	2.00
Limestone	0.90	0.90	0.90	0.90	0.90	0.90
Salt	0.35	0.35	0.35	0.35	0.35	0.35
50% Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
Mineral premix ¹	0.20	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.04	0.22	0.39	0.00	0.00	0.00
DL-HMTBA	0.00	0.00	0.00	0.04	0.25	0.45
L-Lysine HCl, 78%	0.14	0.14	0.14	0.14	0.14	0.14
Vitamin premix ²	0.03	0.03	0.03	0.03	0.03	0.03
Antioxidant	0.03	0.03	0.03	0.03	0.03	0.03
Bentonite	2.00	2.00	2.00	2.00	2.00	2.00
Maifanite	0.41	0.23	0.05	0.40	0.20	0.00
Calculated nutrient levels						
Metabolizable energy, Mcal/kg	2.90	2.91	2.92	2.91	2.91	2.92
Crude protein	21.61	21.71	21.81	21.61	21.71	21.82
Lysine	1.20	1.20	1.20	1.20	1.20	1.20
Methionine	0.37	0.54	0.72	0.37	0.54	0.72
Calcium	0.91	0.91	0.91	0.91	0.91	0.91
Available phosphorus	0.45	0.45	0.45	0.45	0.45	0.45
Methionine + Cystine	0.70	0.88	1.06	0.70	0.88	1.06
Analyzed nutrient levels						
Crude protein	22.09	22.00	22.25	22.50	22.44	22.24
Gross energy, MJ/kg	16.72	16.69	17.02	16.68	17.03	17.02
DL-Methionine	0.41	0.51	0.63	0.37	0.37	0.37
DL-HMTBA	–	–	–	0.03	0.16	0.29
Methionine + Cystine	0.80	0.90	1.01	0.79	0.92	1.03

TSAA = total sulfur amino acids; HMTBA = 2-hydroxy-4-(methylthio)-butanoic acid.

¹ Provide per kilogram of mineral premix: copper, 8 g; iron, 40 g; zinc, 55 g; manganese, 60 g; iodine, 750 mg; selenium, 150 mg; cobalt, 250 mg.

² Provide per kilogram of vitamin premix: vitamin A, 50 million IU; vitamin D₃, 12 million IU; vitamin E, 0.1 million IU; vitamin K₃, 10 g; vitamin B₁, 8 g; vitamin B₂, 32 g; vitamin B₆, 12 g; vitamin B₁₂, 100 mg; nicotinamide, 150 g; D-pantothenic acid, 46 g; folic acid, 5 g; biotin, 500 mg.

isolated from intestinal tissues was reverse transcribed into cDNA using the PrimeScript RT reagent (RR047A, Takara Bio Inc., Kyoto, Japan) according to the manufacturer's guidelines. SybrGreen based quantitative PCR was performed with a quantitative real-time PCR master mix (RR420A, Takara Bio Inc., Kyoto, Japan) in a 7500 real-time PCR system (Applied Biosystems LLC., Massachusetts, America). β -Actin was used as a housekeeping gene and the relative gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used for quantifying selected genes are listed in Table 2.

2.6. Gut morphology

Histomorphology parameters were analyzed by hematoxylin and eosin (H&E) stained tissue sections as described (Frankel et al.,

1993). In brief, the ileal segments fixed in paraformaldehyde were embedded in paraffin and cut into rings of 5 μ m thin slices (Pathology slicer, RM2016, Shanghai Leica Instrument Co., Ltd, Shanghai, China). After processing by H&E staining, the villus and crypt status were observed with light microscopy (Leica DM750, Leica Microsystems, Wetzlar, Hessian, Germany) and measured by randomly selecting 10 intact villi for every bird using Lioo software. Specifically, the villus height was calculated as the distance from the apex of the villus to the junction of the villus and crypt. The crypt depth was calculated as the distance from the junction to the basement membrane of the epithelial cells at the bottom of the crypt. Then, the ratio between villus height and crypt depth was calculated. The average villus height and crypt depth per slide was used as experimental observation.

Table 2
Primer sequences used in real-time quantitative PCR analysis.

Gene	Primer sequence (5'–3')		Product size, bp	Accession number
	Forward	Reverse		
Claudin-1	CATACTCTGGGTCTGGTTGGT	GACAGCCATCCGCATCTTCT	100	NM_001013611.2
Zonula occluden-1	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCATGGCTGGATC	131	XM_040680632.1
Occludin	ACGGCAGCACCTACCTCAA	GGGCGAAGAAGCAGATGAG	123	NM_205128.1
Mucin2	TTCATGATGCTGCTCTTGTTG	CCTGAGCCTTGGTACATTCCTGT	93	XM_040673077.2
Interleukin-1 β	ACTGGGCATCAAGGGCTA	GGTAGAAGATGAAGCGGGTC	131	XM_046931582.1
Interleukin-6	CGCCAGAAATCCCTCCTC	AGGCACTGAAACTCTGGTC	152	NM_204628.2
Interleukin-10	GCTGCCAAGCCCTGTT	CCTCAAACCTCACCTCA	126	NM_001004414.4
β -Actin	CAACACAGTGTCTGTGGTGTAC	CTCCTGCTGTGATCCACATCTG	199	NM_205518.1

Primers were synthesized by Biotech (Shanghai) Co., Ltd.

2.7. Bacterial DNA extraction and sequencing of 16S rRNA

Ileal contents from the same treatment were randomly mixed in pairs to form 5 mixed digesta for the determination of microbiota, and cecal contents were also treated in the same way. Genomic DNA was extracted with the cetyltrimethylammonium ammonium bromide (CTAB) method. The purity and concentration of total DNA were detected by agarose gel electrophoresis. Amplicons from the V3 and V4 regions of the 16S rRNA gene were generated from the extracted DNA using the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The sequencing libraries were constructed by NEBNext Ultra IIDNA Library Prep Kit and quantified by Qubit and Q-PCR. After being confirmed qualified, NovaSeq6000 was used for machine sequencing. Demultiplexed raw sequence data were quality-filtered and denoised using the DADA2 method of QIIME2 to generate amplicon sequence variants. Alpha diversity indices (including Chao1, Shannon, and Observed_otus index) were calculated to evaluate microbial species evenness by QIIME2 software. Beta diversity was evaluated by principal coordinate analysis (PCoA) based on the Bray_curtis distance. Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) was used to identify the differences in the composition of ileal and cecal microbiota. The *t*-test was used to compare the abundances at the genus level. Tax4Fun tools was further used to predict the functional pathway from the 16S rRNA data. STAMP version 2.1.3 (Parks et al., 2014) was also used as a graphical tool.

2.8. Short chain fatty acids

The SCFA concentrations of cecal contents per replicate from each treatment were measured by gas chromatograph (GC-2014; Shimadzu Corporation, Kyoto, Japan) equipped with a hydrogen flame detector and a capillary column (Agilent Technologies, Santa Clara, CA, USA; 30 m long, 0.32-mm diameter, 0.50- μ m film thickness). In brief, 0.4 g of thawed cecal contents were weighed into a 2-mL sterile centrifuge tube. After adding 0.5 mL deionized water, the mixture was homogenized with a vortex and then centrifuged at 4 °C, 25,155 \times g for 10 min. Then, 0.2 mL of supernatant was collected and transferred into a new 1.5-mL microcentrifuge tube containing 50 μ L of metaphosphoric acid (25%, wt/vol) containing

2-ethylbutyric acid as the internal standard. After that, the mixed solution was cultured at 4 °C overnight to precipitate protein and then centrifuged for 10 min at 25,155 \times g. Finally, 0.4 μ L of supernatant was injected into the gas chromatograph for analysis. Results are expressed as micro mol per gram of the weight of digesta.

2.9. Statistical analysis

For comparing the main effects of sources and levels and their interaction, statistical differences were determined by 2-way ANOVA in a 2 \times 3 factorial arrangement with Duncan's test for multiple comparisons. Meanwhile, one-way ANOVA and Duncan's multiple comparisons were used when a significant interaction was observed. All analysis were carried out with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant and $0.05 < P < 0.10$ was viewed as a trend.

3. Results

3.1. Intestinal epithelial barrier

No statistically significant differences ($P > 0.05$) were observed in the relative mRNA expressions of occludin and mucin2 in the ileum of broilers in response to the sources and levels of methionine (Table 3). However, there was a significant increase ($P < 0.05$) in the relative mRNA expression of claudin-1 in the DL-HMTBA treatment on d 14 and 21. In addition, compared with middle- and high-TSAA treatments, low-TSAA treatment increased the relative mRNA expression of zonula occluden-1 (*ZO-1*) on d 14 ($P < 0.05$). Accordingly, in comparison with the DL-methionine treatment, dietary DL-HMTBA supplementation decreased the serum DAO content on d 14 and 21 ($P < 0.01$) (Table 4).

3.2. The mRNA expression levels of inflammatory cytokines in the ileum

As shown in Table 5, dietary supplementation with DL-HMTBA downregulated ($P < 0.05$) the relative mRNA expression of interleukin-6 (*IL-6*) in the ileum on d 14 and 21. Besides, compared to the middle- and high-TSAA treatments, low-TSAA treatment increased the relative mRNA expression of interleukin-1 β (*IL-1 β*)

Table 3
Effects of different dietary sources and levels of methionine on mRNA expression of epithelial barrier related genes in ileum ($n = 10$).

Item	Groups ¹	14 d				21 d			
		Claudin-1	ZO-1	Occludin	Mucin2	Claudin-1	ZO-1	Occludin	Mucin2
DL-Methionine	Low	1.04	1.21	0.95	0.65	1.09	0.99	1.09	0.94
	Middle	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	High	0.90	1.03	0.90	0.83	0.99	0.93	1.08	1.15
DL-HMTBA	Low	1.42	1.46	0.92	0.91	1.23	1.00	1.12	0.98
	Middle	1.16	0.94	1.00	0.97	1.42	0.96	0.90	0.81
	High	0.90	0.98	1.07	1.09	1.05	0.85	0.95	0.93
SEM		0.05	0.04	0.04	0.05	0.05	0.03	0.03	0.04
Main effect									
Sources	DL-Methionine	0.98 ^b	1.08	0.95	0.84	1.02 ^b	0.97	1.06	1.04
	DL-HMTBA	1.15 ^a	1.13	0.99	0.99	1.23 ^a	0.94	0.99	0.91
Levels	Low	1.23 ^a	1.34 ^a	0.94	0.80	1.17	1.00	1.10	0.96
	Middle	1.08 ^{ab}	0.97 ^b	1.00	0.99	1.22	0.98	0.95	0.91
	High	0.90 ^b	1.00 ^b	0.98	0.96	1.02	0.89	1.01	1.04
P-value									
Sources		0.04	0.52	0.57	0.11	0.05	0.53	0.21	0.09
Levels		0.01	<0.01	0.78	0.23	0.26	0.31	0.08	0.31
Sources \times Levels		0.18	0.14	0.49	0.39	0.29	0.84	0.45	0.26

HMTBA = 2-hydroxy-4-(methylthio)-butanoic acid; ZO-1 = zonula occluden-1.

Means within a column without a common superscript differ significantly ($P < 0.05$).

¹ Low = 80% total sulfur amino acid; Middle = 100% total sulfur amino acid; High = 120% total sulfur amino acid.

Table 4Effects of different dietary sources and levels of methionine on the concentrations of diamine oxidase in serum of broilers (ng/mL, $n = 10$).

Item	Groups ¹	14 d	21 d
DL-Methionine	Low	17.77	14.61
	Middle	16.73	15.15
	High	15.87	14.86
DL-HMTBA	Low	15.06	13.24
	Middle	14.41	12.11
	High	15.66	13.81
SEM		0.29	0.31
Main effect			
Sources	DL-Methionine	16.79 ^a	14.87 ^a
	DL-HMTBA	15.04 ^b	13.05 ^b
Levels	Low	16.41	13.92
	Middle	15.57	13.63
	High	15.77	14.33
P-value			
Sources		<0.01	<0.01
Levels		0.39	0.62
Sources × Levels		0.12	0.33

HMTBA = 2-hydroxy-4-(methylthio)-butanoic acid.

Means within a column without a common superscript differ significantly ($P < 0.05$).¹ Low = 80% total sulfur amino acid; Middle = 100% total sulfur amino acid; High = 120% total sulfur amino acid.

and *IL-6* on d 14 ($P < 0.05$). Compared to the high dosage, low- and middle-TSAA treatments increased the relative mRNA expression of *IL-1 β* on d 21 ($P < 0.05$). In addition, there was a significant interaction between sources and levels on the expression of interleukin-10 (*IL-10*) ($P < 0.05$). Specifically, compared with the middle- and high-level TSAA groups, the expression of *IL-10* in the low-level TSAA group increased when the methionine source was DL-HMTBA ($P < 0.05$) in 14-d-old broilers, but the expression was not affected by different levels of DL-methionine. In 21-d-old broilers, the mRNA expression of *IL-10* in the low-level TSAA group was higher than that of the high-TSAA group when the source was DL-methionine ($P < 0.05$), but the expression was not affected by different levels of DL-HMTBA.

3.3. Ileal morphology

The results of histomorphology parameters of ileum in 14-d-old broilers are presented in Supplementary Fig. S1 and Table 6. Dietary

supplementation with DL-HMTBA significantly increased villus height ($P < 0.05$) but had no significant effects on crypt depth. Besides, compared to the middle-TSAA treatment, the high-TSAA treatment decreased villus height ($P < 0.05$), and the low-TSAA treatment increased crypt depth ($P < 0.05$). There was a significant interaction between sources and levels on the villus height to crypt depth ratio ($P < 0.05$). Specifically, compared with low- and high-TSAA treatments, the middle-level TSAA of DL-HMTBA treatment significantly increased villus height to crypt depth ratio ($P < 0.05$), but this parameter was not influenced by different levels of DL-methionine.

For the 21-d-old broilers, the results are shown in Supplementary Fig. S2 and Table 6. The dietary supplementation of middle-level TSAA significantly decreased the crypt depth and increased the villus height to crypt depth ratio compared with the other 2 levels ($P < 0.05$). With regard to crypt depth, there was no apparent difference between the 2 sources ($P > 0.05$), while a decreasing tendency for DL-HMTBA treatment was observed ($P = 0.09$).

3.4. Ileal microbiota composition

The effects of different dietary sources and levels of methionine on the ileal microbiota composition of broilers on d 14 are shown in Fig. 1. Chao1, Shannon, and Observed_otus indices were used to estimate the alpha diversity. There were no differences observed in middle- and high-TSAA groups, but the supplementation of DL-HMTBA increased Chao1 and Observed_otus indexes ($P < 0.05$) and showed a tendency to increase Shannon index ($P = 0.051$) in comparison with DL-methionine when birds were on low-TSAA treatment (Fig. 1A). However, beta diversity results (PCoA analysis) indicated that there were no distinct differences between the microbiota of the DL-HMTBA group and the DL-methionine group at 3 TSAA levels (Fig. 1B). The abundance of ileal microbial species distribution in phylum (Fig. 1C) and genus (Fig. 1D) among treatment groups was shown. The microorganisms in the ileum were mainly Firmicutes and Proteobacteria. Overall, the relative abundance of *Lactobacillus* increased with increasing TSAA levels. At the genus level (Fig. 1E), *Faecalibacterium*, *Ruminococcus_torques_group*, *Eubacterium_coprostanoligenes_group*, *UCG-005*, *Paludicola*, *Incertae_Sedis*, *Fournierella*, *Oscillospiraceae*, and

Table 5Effects of different dietary sources and levels of methionine on mRNA expression of inflammation related genes in ileum ($n = 10$).

Item	Groups ¹	14 d			21 d		
		<i>IL-1β</i>	<i>IL-6</i>	<i>IL-10</i>	<i>IL-1β</i>	<i>IL-6</i>	<i>IL-10</i>
DL-Methionine	Low	1.86	1.34	1.04 ^b	1.04	0.98	1.26 ^a
	Middle	1.00	1.00	1.00 ^b	1.00	1.00	1.00 ^{ab}
	High	0.90	0.98	0.91 ^{bc}	0.69	0.80	0.87 ^b
DL-HMTBA	Low	2.48	0.99	1.53 ^a	0.86	0.82	0.83 ^b
	Middle	1.07	0.84	0.89 ^{bc}	0.96	0.68	0.93 ^b
	High	0.79	0.87	0.66 ^c	0.65	0.64	0.96 ^{ab}
SEM		0.11	0.04	0.05	0.05	0.05	0.04
Main effect							
Sources	DL-Methionine	1.23	1.10 ^a	0.99	0.91	0.93 ^a	1.03
	DL-HMTBA	1.41	0.90 ^b	1.04	0.82	0.71 ^b	0.91
Levels	Low	2.17 ^a	1.16 ^a	1.29 ^a	0.95 ^a	0.90	1.04
	Middle	1.03 ^b	0.93 ^b	0.94 ^b	0.98 ^a	0.84	0.96
	High	0.85 ^b	0.92 ^b	0.78 ^b	0.67 ^b	0.71	0.91
P-value							
Sources		0.27	0.01	0.61	0.42	0.02	0.10
Levels		<0.01	0.03	<0.01	0.04	0.25	0.45
Sources × Levels		0.22	0.46	<0.01	0.82	0.71	0.04

IL-1 β = interleukin-1 β ; *IL-6* = interleukin-6; *IL-10* = interleukin-10; HMTBA = 2-hydroxy-4-(methylthio)-butanoic acid.Means within a column without a common superscript differ significantly ($P < 0.05$).¹ Low = 80% total sulfur amino acid; Middle = 100% total sulfur amino acid; High = 120% total sulfur amino acid.

Table 6Effects of different dietary sources and levels of methionine on histomorphology parameters of ileum in broilers ($n = 10$).

Item	Group ¹	14 d			21 d		
		VH, μm	CD, μm	VH:CD ratio	VH, μm	CD, μm	VH:CD ratio
DL-Methionine	Low	471.5	165.1	2.87 ^b	651.3	197.3	3.42
	Middle	448.6	154.5	3.15 ^b	692.1	185.7	3.91
	High	406.4	143.4	2.96 ^b	716.7	219.4	3.43
DL-HMTBA	Low	487.0	166.7	3.04 ^b	679.7	198.2	3.50
	Middle	523.0	131.4	3.99 ^a	699.1	164.3	4.28
	High	435.3	151.9	3.14 ^b	668.5	200.5	3.45
SEM		10.5	3.4	0.06	11.6	4.3	0.08
Main effect							
Sources	DL-Methionine	440.9 ^b	153.6	2.98 ^b	686.7	200.8	3.58
	DL-HMTBA	480.3 ^a	150.6	3.35 ^a	682.3	187.2	3.73
Levels	Low	479.7 ^a	166.0 ^a	2.96 ^b	665.5	197.7 ^a	3.46 ^b
	Middle	485.8 ^a	143.0 ^b	3.57 ^a	695.4	175.0 ^b	4.08 ^a
	High	420.9 ^b	147.7 ^b	3.04 ^b	693.9	210.5 ^a	3.44 ^b
P-value							
Sources		0.05	0.48	<0.01	0.86	0.09	0.23
Levels		0.01	0.01	<0.01	0.51	<0.01	<0.01
Sources \times Levels		0.44	0.10	<0.01	0.40	0.44	0.52

VH = villus height; CD = crypt depth; HMTBA = 2-hydroxy-4-(methylthio)-butanoic acid.

Means within a column without a common superscript differ significantly ($P < 0.05$).¹ Low = 80% total sulfur amino acid; Middle = 100% total sulfur amino acid; High = 120% total sulfur amino acid.

Oscillibacter were enriched in the ileum of DL-HMTBA-low level TSAA treated group. Taxa abundancies were further analyzed by LEfSe (LDA > 2.0; Fig. 1F). Families such as Streptococcaceae, Pseudomonadaceae, and Burkholderiaceae were enriched; at the genus level, *Streptococcus*, *Pseudomonas*, and *Ralstonia* were increased in DL-methionine-low TSAA treated group. *Eubacterium_hallii_group* and *CHKC1001* were enriched in DL-HMTBA-middle TSAA treated group. While Lactobacillaceae and Enterococcaceae were enriched in DL-HMTBA-high TSAA treated group.

Fig. 2 shows the effects of different sources and levels of methionine on the ileal microbiota composition of broilers on d 21. In the alpha diversity indexes (Fig. 2A), the addition of DL-HMTBA in high-TSAA treatment showed a tendency to decrease Chao1 ($P = 0.075$) and Observed_otus ($P = 0.074$) indexes, while the addition of DL-HMTBA in low-level TSAA group showed a tendency to increase Shannon index ($P = 0.063$). The PCoA analysis showed that treatment groups were not significantly distinguished from each other (Fig. 2B). In terms of the bacteria composition, dietary supplementation of DL-HMTBA significantly increased the relative abundance of the Firmicutes phyla (Fig. 2C). At the genus level, DL-HMTBA treated group significantly reduced the relative abundance of *Streptococcus* but increased the relative abundance of *Lactobacillus* (Fig. 2D). The statistical analysis performed at the genus level showed that supplementation of DL-HMTBA only increased the relative abundance of *Lactobacillus* in low-TSAA treatment (Fig. 2E) and the relative abundance of *Blautia* in high-TSAA treatment (Fig. 2F). The differential analysis on gut bacteria showed that DL-methionine-low TSAA treated group had a higher abundance of *Clostridium_sensu_stricto_1*, and the DL-methionine-high TSAA treated group had the highest abundance of *Lactobacillus_cacaonum*, Campylobacteria, and *Lachnospiraceae_NK4A136_group*, while the DL-HMTBA-low TSAA treated group had the highest abundance of Halomonadaceae (Fig. 2G).

3.5. Functional prediction analysis

The Tax4Fun software was further used to predict the microbial functional categories of 21 d ileum. As shown in Fig. 3A, the gene functions associated with membrane transport in DL-HMTBA-low TSAA treated group were significantly lower than that in DL-methionine group. Moreover, 4 differentially abundant KEGG

pathways were found between the 2 groups in the high-TSAA treatment ($P < 0.05$, Fig. 3B). Among them, the microbial gene functions related to drug resistance and enzyme families were much higher in the ileal microbiome of the DL-methionine group. In contrast, the abundance of pathways related to endocrine and metabolic diseases and biosynthesis of other secondary metabolites decreased in DL-methionine group.

3.6. Cecal microbiota composition

The 16S DNA of intestinal bacteria in the cecum of broilers at 14 and 21 d of age was also subjected to sequencing. The effects of different dietary sources and levels of methionine on the cecal microbiota composition of broilers on d 14 and 21 are shown in Figs. 4 and 5, respectively. No significant differences in alpha diversity (Figs. 4A and 5A) and beta diversity (Figs. 4B and 5B) of cecal microbiota were found. The abundance of cecal microbial species distribution in phylum (Figs. 4C and 5C) and genus (Figs. 4D and 5D) among treatment groups are shown.

For 14-d-old broilers, at the genus level, *Candidatus_Arthromitus* was enriched in the cecum of the DL-methionine group in low-TSAA treatment ($P < 0.05$; Fig. 4E) and in the DL-HMTBA group in middle-TSAA treatment ($P < 0.05$; Fig. 4F). Besides, in middle-TSAA treatment, *Clostridia_vadinBB60_group* was increased when DL-methionine was supplemented. Meanwhile, the relative abundance of *Lachnospiraceae_UCG-010* showed a significant increase in DL-HMTBA-high TSAA treated group ($P < 0.05$; Fig. 4G). The results of the LEfSe analysis of the microbiota were shown in Fig. 4H. In this regard, DL-methionine-low TSAA treated group had a higher abundance of Vampirivibrionia, Gastranaerophilales, *Candidatus_Arthromitus*, Clostridiales, and Clostridiaceae, and the DL-methionine-high TSAA treated group had the highest abundance of *Ruminococcus_sp.* Proteobacteria such as Enterobacteriaceae (*Escherichia_Shigella* and *Proteus*) and Morganellaceae were significantly increased as well as *Anaerofilum* in the DL-HMTBA-low TSAA treated group. The DL-HMTBA-middle TSAA treated group had the highest abundance of *Tyzzrella*, *Anaerotignum_lactatifermentans*, *Oscillibacter*, *Blautia*, and *Lachnospiraceae_UCG_010*, whereas microbiota such as Pseudonocardiaceae, *Flavonifractor* were significantly enriched in the DL-HMTBA-high TSAA treated group.

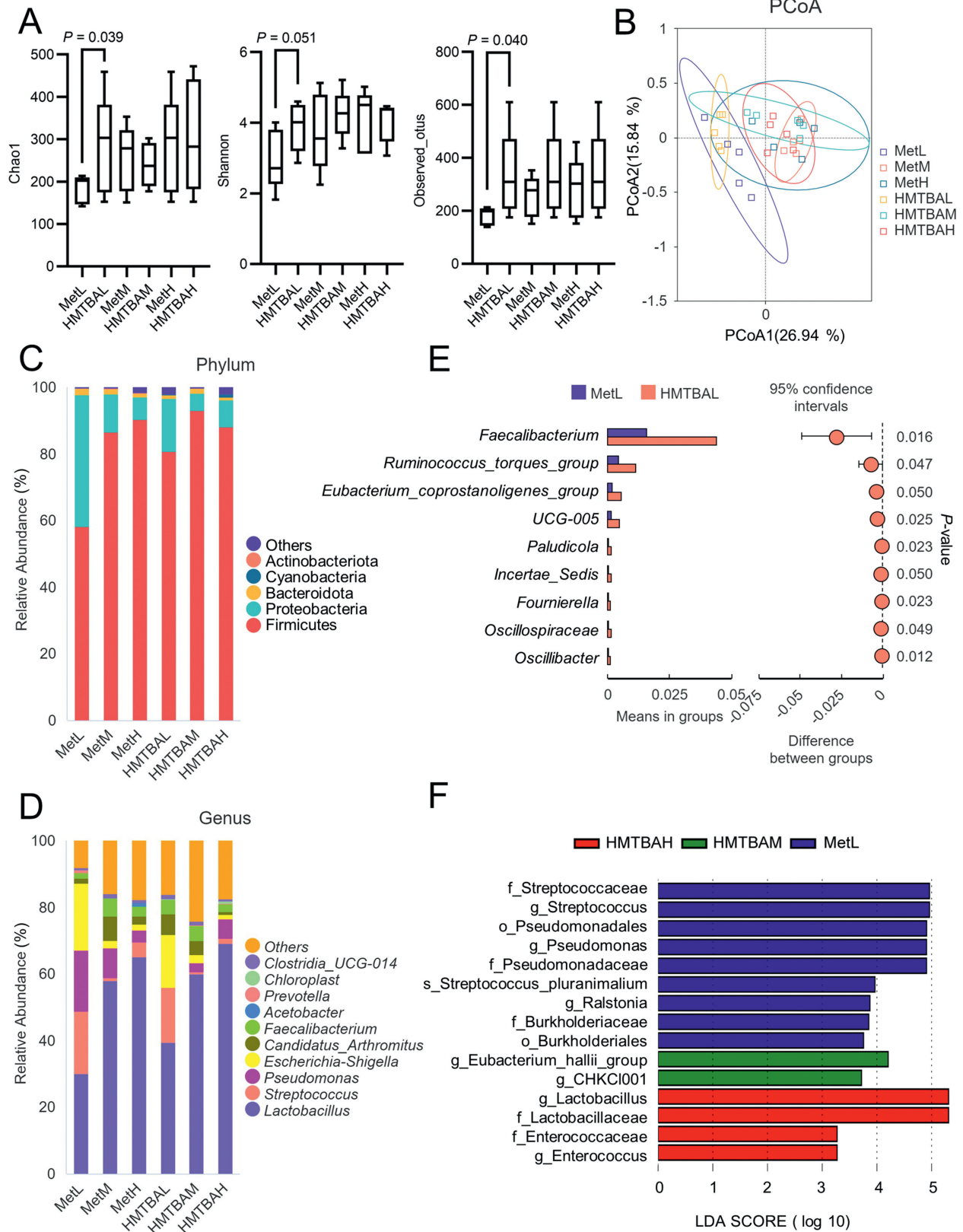


Fig. 1. Effects of different dietary sources and levels of methionine on ileal microbiota composition on d 14. (A) Three different alpha diversity metrics. (B) Principal coordinate analysis of Bray–Curtis distances of the ileum samples from 6 treatments. (C, D) Gut microbiota composition (relative abundance, %) at phylum and genus level. The phylum level shows the top 5 species in total abundance, and the genus level shows the top 10 species in total abundance. (E) The *t*-test results at the genus level. (F) The LefSe analysis of the ileal microbiota. MetL = DL-methionine-80% total sulfur amino acid (TSAA) group; MetM = DL-methionine-100% TSAA group; MetH = DL-methionine-120% TSAA group; HMTBAL = DL-2-hydroxy-4-(methylthio)-butanoic acid (DL-HMTBA)-80% TSAA group; HMTBAM = DL-HMTBA-100% TSAA group; HMTBAH = DL-HMTBA-120% TSAA group.

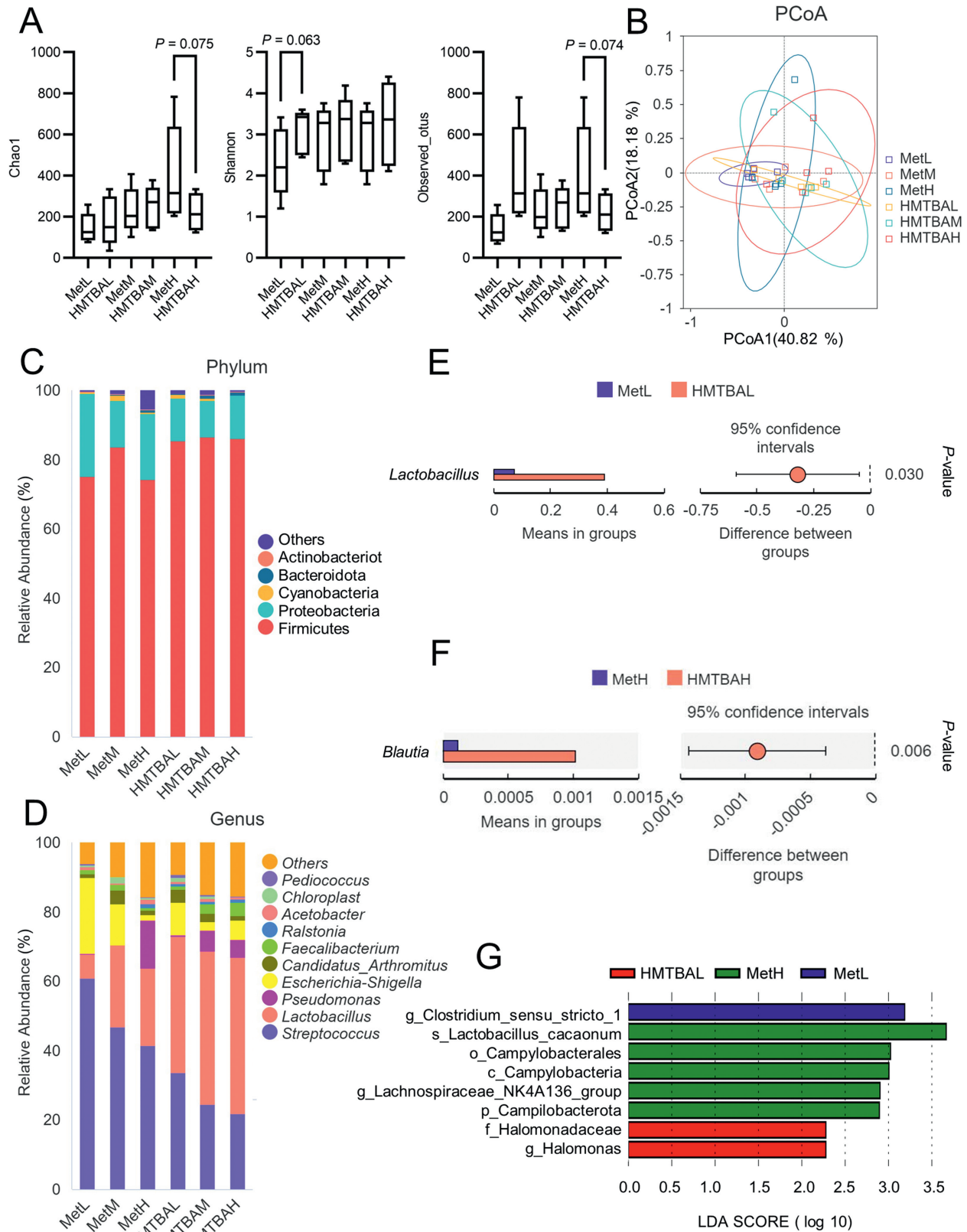


Fig. 2. Effects of different dietary sources and levels of methionine on ileal microbiota composition on d 21. (A) Three different alpha diversity metrics. (B) Principal coordinate analysis of Bray–Curtis distances of the ileum samples from 6 treatments. (C, D) Gut microbiota composition (relative abundance, %) at phylum and genus level. The phylum level shows the top 5 species in total abundance, and the genus level shows the top 10 species in total abundance. (E, F) The *t*-test results at the genus level. (G) The LEfSe analysis of the ileal microbiota. MetL = DL-methionine-80% total sulfur amino acid (TSAA) group; MetM = DL-methionine-100% TSAA group; MetH = DL-methionine-120% TSAA group; HMTBAL = DL-2-hydroxy-4-(methylthio)-butanoic acid (DL-HMTBA)-80% TSAA group; HMTBAM = DL-HMTBA-100% TSAA group; HMTBAH = DL-HMTBA-120% TSAA group.

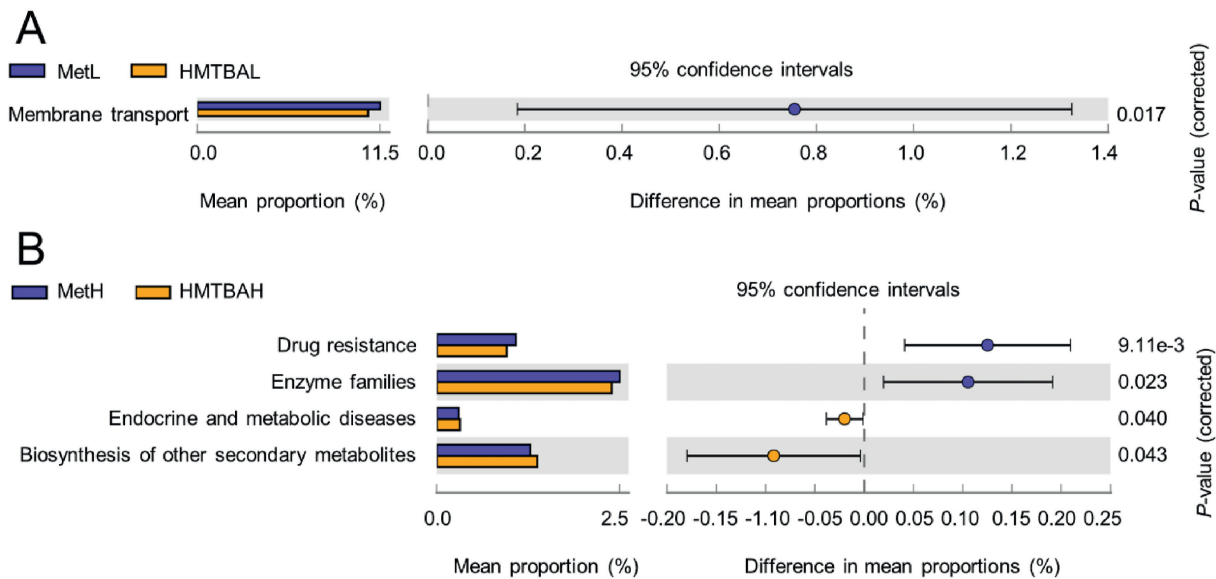


Fig. 3. Functional prediction analysis of 21 d ileal microbiota. Tax4Fun analysis was used to predict Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways. Significant KEGG pathways for the ileal microbiome of the 2 groups at the same total sulfur amino acid (TSAA) level were identified by STAMP software. MetL = DL-methionine-80% TSAA group; MetH = DL-methionine-120% TSAA group; HMTBAL = DL-2-hydroxy-4-(methylthio)-butanoic acid (DL-HMTBA)-80% TSAA group; HMTBAH = DL-HMTBA-120% TSAA group.

In 21-d-old broilers, *Blautia* was more abundant in the DL-methionine group when TSAA was offered in low dosage ($P < 0.05$; Fig. 5E). In addition, *Intestinimonas* and *Clostridium_sensu_stricto_1* were more abundant, whereas *Subdoligranulum*, *Tyzzerella*, *Flavonifractor*, and *Lachnospiraceae_UCG-010* showed a decreased condition in the DL-HMTBA group when TSAA was offered in middle dosage ($P < 0.05$; Fig. 5F). LEfSe analysis further demonstrated the differential microbial taxa among the 6 groups. As shown in Fig. 5G, *Acetobacter*, and *Bacillus* were significantly enriched in the DL-methionine-high TSAA treated group. In the meantime, *Clostridium_sensu_stricto_1* was significantly enriched in DL-HMTBA-low TSAA treated group and the DL-HMTBA-high TSAA treated group had the highest abundance of *Mordavella_sp_* and *Oscillospira*.

3.7. Cecal short chain fatty acids profile

No significant interactions were observed on the profile of SCFA in the cecal content of birds (Table 7). Notably, DL-HMTBA supplementation significantly elevated the concentrations of acetate, propionate, butyrate, and total SCFA ($P < 0.01$), but did not affect the concentrations of isobutyrate, isovalerate, and valerate. The profile of SCFA also indicates that high-TSAA treatment had higher concentrations of isobutyrate and isovalerate, but a lower concentration of butyrate compared with the middle-TSAA group ($P < 0.05$).

4. Discussion

Adequate dietary supply of amino acids is a key determinant of gut health and functions (Beaumont and Blachier, 2020). Accumulating evidence has shown that methionine is closely correlated with the intestinal health of animals (Bauchart-Thevret et al., 2009; Chen et al., 2014; Ramalingam et al., 2010; Saito et al., 2017). However, the effects of methionine sources and levels on the intestinal health of broiler chicks remain unclear. Thus, physical, immunological, and microbiological barriers including intestinal morphology and SCFA levels were investigated for this unresolved question. In this study, we demonstrated that DL-HMTBA supported

better gut health of broilers under normal feeding conditions and had no difference in growth performance compared with DL-methionine. However, TSAA level had effect on the growth performance of chickens. Moreover, the methionine requirement is not consistent for different intestinal health dimensions.

During all stages of animal's growth, the intestinal physical barrier plays an important role to regulate IP. In addition, disruption of the intestinal barrier and increased IP may play a role in many pathological or inflammatory states (Khoshbin and Camilleri, 2020). The intestinal physical barrier is primarily composed of intestinal mucosal epithelial cells and their TJ. The passage of ions and molecules through the paracellular pathway in epithelial and endothelial cells is regulated by TJ, where their degree of sealing varies according to external stimuli, physiological and pathological conditions (Gonzalez-Mariscal et al., 2008). Several proteins such as claudin-1 and ZO-1 are involved in the formation of TJ in poultry (Kimura et al., 1996; Simard et al., 2005) and their relative mRNA expressions depict the IP of the gut. Besides, IP can also be measured indirectly through biomarkers. DAO is an extensively used biomarker for IP determination in mammals (Cakmaz et al., 2013) and poultry (Lei et al., 2013), which is synthesized mainly by intestinal mucosal cells and is a highly active intracellular enzyme. Notably, lower DAO serum levels are associated with decreased IP. In this study, we observed that DL-HMTBA treatment increased the gene expression of claudin-1 in the ileum, suggesting that DL-HMTBA had a positive effect on the physical barrier of broilers. Further, we also noticed that supplementation of DL-HMTBA significantly reduced the serum DAO content supporting the gene expression data and collectively indicating decreased IP. These results are consistent with the previous work of Martin-Venegas (Martin-Venegas et al., 2013). It is worth noting that DL-HMTBA is chemically an organic acid for which acidifying functions have been reported in the literature (Batonon-Alavo et al., 2016). Many authors have confirmed that dietary supplemented mixed organic acid improved the health status of broilers by enhancing the TJ proteins expression (Ma et al., 2021; Melaku et al., 2021). Therefore, we deduced that DL-HMTBA as a source will have a more positive effect in terms of the intestinal physical barrier,

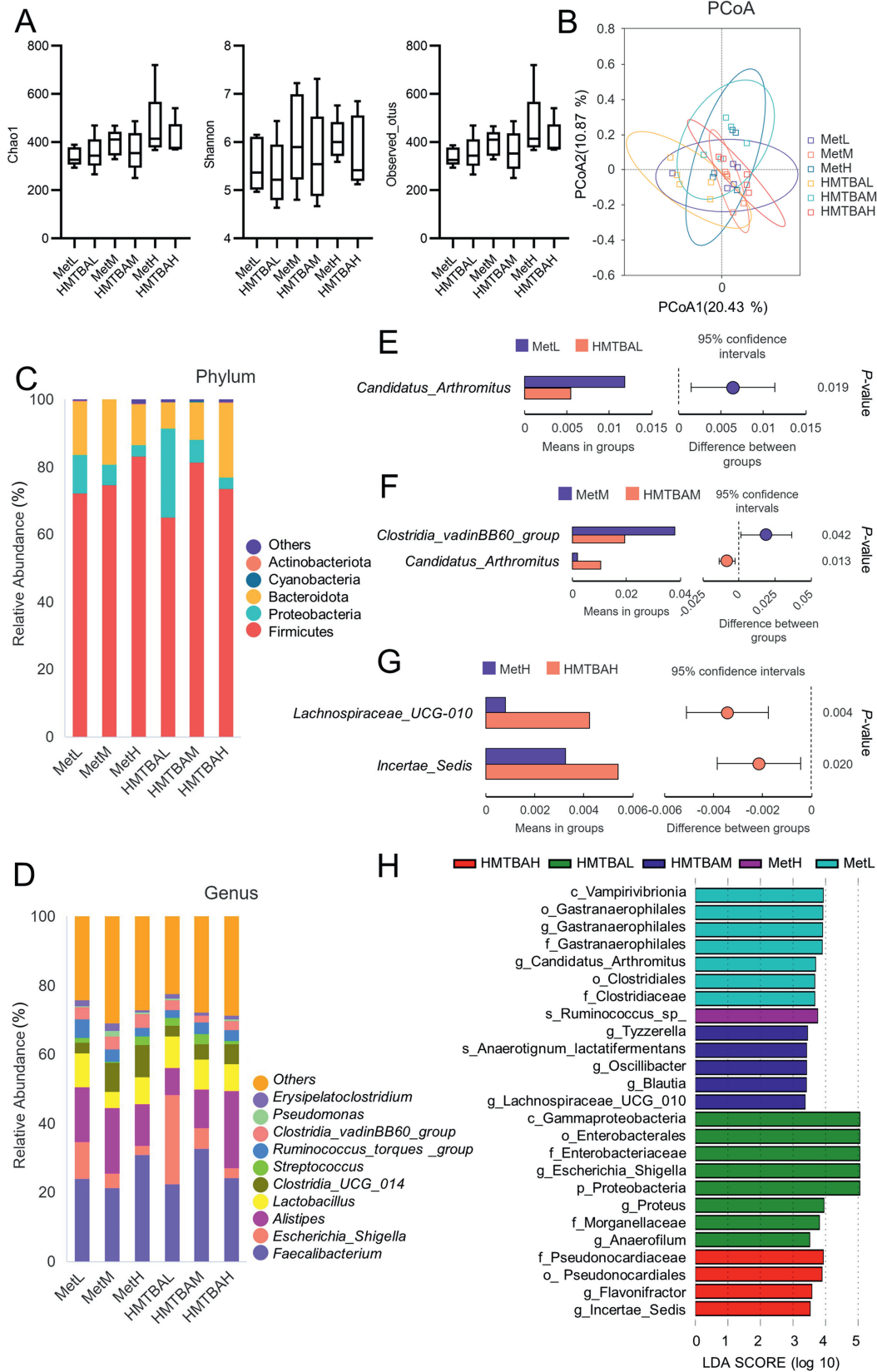


Fig. 4. Effects of different dietary sources and levels of methionine on cecal microbiota composition on d 14. (A) Three different alpha diversity metrics. (B) Principal coordinate analysis of Bray–Curtis distances of the cecal samples from 6 treatments. (C, D) Gut microbiota composition (relative abundance, %) at phylum and genus level. The phylum level shows the top 5 species in total abundance, and the genus level shows the top 10 species in total abundance. (E–G) The *t*-test results at the genus level. (H) The LEfSe analysis of the cecal microbiota. MetL = DL-methionine-80% total sulfur amino acid (TSAA) group; MetM = DL-methionine-100% TSAA group; MetH = DL-methionine-120% TSAA group; HMTBAL = DL-2-hydroxy-4-(methylthio)-butanoic acid (DL-HMTBA)-80% TSAA group; HMTBAM = DL-HMTBA-100% TSAA group; HMTBAH = DL-HMTBA-120% TSAA group.

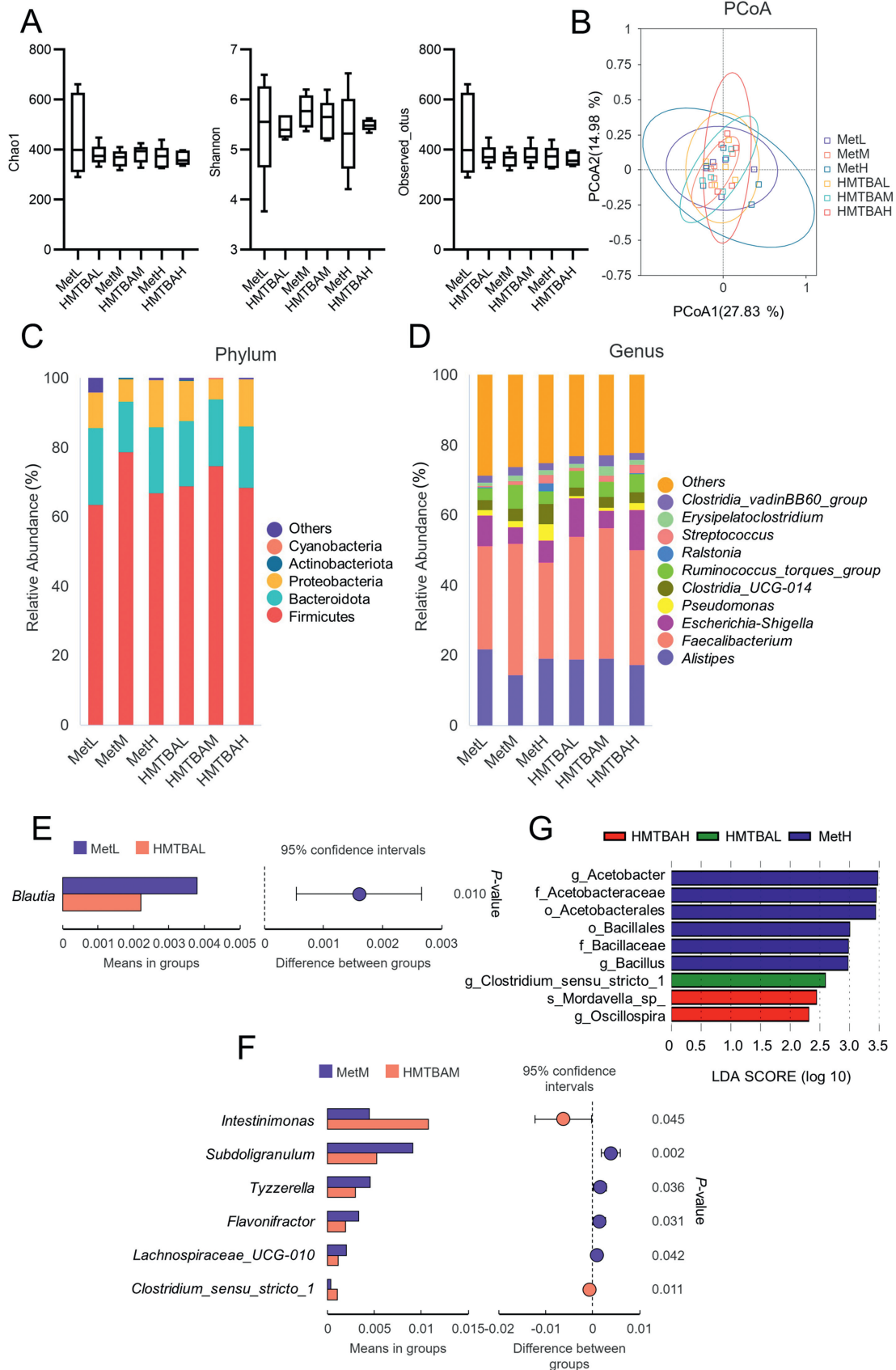


Fig. 5. Effects of different dietary sources and levels of methionine on cecal microbiota composition on d 21. (A) Three different alpha diversity metrics. (B) Principal coordinate analysis of Bray–Curtis distances of the cecal samples from 6 treatments. (C, D) Gut microbiota composition (relative abundance, %) at phylum and genus level. The phylum level shows the top 5 species in total abundance, and the genus level shows the top 10 species in total abundance. (E, F) The *t*-test results at the genus level. (G) The LEfSe analysis of the cecal microbiota. MetL = DL-methionine-80% total sulfur amino acid (TSAA) group; MetM = DL-methionine-100% TSAA group; MetH = DL-methionine-120% TSAA group; HMTBAL = DL-2-hydroxy-4-(methylthio)-butanoic acid (DL-HMTBA)-80% TSAA group; HMTBAM = DL-HMTBA-100% TSAA group; HMTBAH = DL-HMTBA-120% TSAA group.

Table 7Effects of different dietary sources and levels of methionine on the concentrations of the short chain fatty acids in the cecum on d 21 ($\mu\text{mol/g}$, $n = 10$).

Item	Group ¹	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Total acid
DL-Methionine	Low	16.32	1.32	0.22	5.39	0.34	0.34	27.16
	Middle	14.21	1.23	0.17	3.96	0.20	0.19	19.96
	High	11.97	1.39	0.34	3.07	0.55	0.38	20.66
DL-HMTBA	Low	21.40	2.22	0.24	6.17	0.31	0.32	30.69
	Middle	21.77	1.70	0.22	6.71	0.18	0.30	31.14
	High	19.67	1.79	0.34	4.70	0.34	0.31	27.33
SEM		0.84	0.10	0.02	0.31	0.04	0.03	0.98
Main effect								
Sources	DL-Methionine	14.17 ^b	1.31 ^b	0.24	4.14 ^b	0.35	0.30	22.20 ^b
	DL-HMTBA	20.99 ^a	1.92 ^a	0.27	5.86 ^a	0.27	0.31	29.80 ^a
Levels	Low	18.86	1.80	0.23 ^b	5.78 ^a	0.32 ^{ab}	0.33	29.24
	Middle	17.99	1.45	0.19 ^b	5.34 ^a	0.19 ^b	0.24	25.55
	High	15.62	1.58	0.34 ^a	3.89 ^b	0.45 ^a	0.34	24.19
P-value								
Sources		<0.01	<0.01	0.62	<0.01	0.21	0.90	<0.01
Levels		0.22	0.43	0.02	0.02	0.01	0.30	0.06
Sources \times Levels		0.71	0.51	0.84	0.34	0.48	0.36	0.15

HMTBA = 2-hydroxy-4-(methylthio)-butanoic acid.

Means within a column without a common superscript differ significantly ($P < 0.05$).¹ Low = 80% total sulfur amino acid; Middle = 100% total sulfur amino acid; High = 120% total sulfur amino acid.

which may be partly related to the fact that it is an organic acid in nature.

The gastrointestinal tract is the largest immune organ and is constantly exposed to a variety of antigens from diets, commensal bacteria, and pathogens (Takiishi et al., 2017). When faced with antigens, the intestinal tract not only needs to be protected by a physical barrier but also should have a robust host defense through the mucosal immune system (Pelaseyed et al., 2014). Numerous studies have confirmed that methionine is involved in animal immune functions (Ji et al., 2019; Kalvandi et al., 2019; Montout et al., 2021; Wu et al., 2012). For example, Ji et al. (2019) indicated that methionine treatment attenuated the LPS-induced inflammation in RAW 264.7 and macrophages were associated with the methionine-derivative SAM-mediated DNA methylation (Ji et al., 2019). In stress conditions such as heat stress, dietary supplementation with methionine is vital to improve the immune and antioxidant status and growth performance of animals (Kalvandi et al., 2019). On the contrary, methionine deficiency could impair the cellular immune function of broilers (Wu et al., 2012). Immune cells which are present in the lamina propria or intermingled in the monolayer of epithelial cells, together with epithelial cells and myofibroblasts secrete cytokines, which regulate both innate and adaptive immune responses to maintain the balance in the intestine (Mahapatro et al., 2021). In the present study, we further investigated the role of methionine on the intestinal immune barrier of broilers by analyzing the expressions of several inflammatory cytokines. IL-1 β and IL-6 are commonly recognized as mucosal pro-inflammatory cytokines. Studies have demonstrated that the expressions of IL-1 β and IL-6 would increase in response to intestinal insults, such as challenges with *Eimeria maxima* (Hong et al., 2006) and *C. perfringens* (Fasina and Lillehoj, 2019; Zhang et al., 2019). Notably, in our study, the supplementation of DL-HMTBA significantly downregulated the expression of IL-6. Some other studies also demonstrated the positive effects of DL-HMTBA on immune response (Dahiya et al., 2007; Tykalowski et al., 2019), even though the underlying mechanism of action is yet to be elucidated. It has been reported that low-dose methionine supplementation decreased the humoral immunocompetence of broiler chickens irrespective of the methionine source i.e. DL-methionine (Wu et al., 2018) or DL-HMTBA (Zhang and Guo, 2008). Our results consistently demonstrated that low-TSAA treatment increased the expression of IL-1 β and IL-6. Similar

results were observed by Matthews et al. (2021) who reported that genetic expression of IL-1 β and IL-6 were significantly higher in methionine and choline deficient diet-fed mice compared with those in chow diet-fed mice (Matthews et al., 2021). Future work should focus on measuring the full spectrum of inflammatory cytokines but with reasonable certainty, we can argue that adequate methionine is required for a modest immune response.

A robust intestinal architecture (higher villus height to crypt depth ratio) plays a key role to guarantee better growth performance and intestinal homeostasis of broilers (Xu et al., 2003). Villi are the main site of nutrient absorption, and a large crypt indicates rapid tissue turnover and high demand for new tissue (Stojanovic et al., 2021). Interestingly, the methionine source effect was observed where DL-HMTBA increased the ileal villus height and villus height to crypt depth ratio on d 14. A similar beneficial effect was also demonstrated in piglets (Li et al., 2014). Additionally, a limited number of studies have also shown that methionine supplementation could improve villus architecture either by in ovo injection (Dang et al., 2022) or direct supplementation to piglets (Chen et al., 2014). Our findings also indicated that the middle-level TSAA group had a higher villus height to crypt depth ratio suggesting better intestinal architecture at this TSAA level.

Alpha diversity and beta diversity are the most used indicators to assess intestinal microbial diversity. In general, alpha diversity and beta diversity show intra-sample and inter-sample differences, respectively. In the ileum of d 14, our results revealed that low-TSAA supplementation of DL-HMTBA resulted in more species with low abundance, more observed species, higher microbial diversity, and more uniform species distribution. However, the methionine level but not the source had an impact on the beta diversity. It is worth noting that methionine source and level significantly affected the community structure and composition of the ileal microbiome. We speculate that this may be related to the consensus that the small intestine is the major site of absorption for DL-methionine and DL-HMTBA. Interestingly, the relative abundance of *Lactobacillus* was increased with methionine level in 14 d ileum, but methionine source had a greater effect on this analysis item on d 21. Previous findings from our lab showed that the key stage of species composition shift is 14 to 21 d during mucosal bacterial community succession of broiler chickens (unpublished data), which might be the probable explanation for these results. To be specific, DL-HMTBA increased the relative abundance of

Lactobacillus in 21 d ileum. Similarly, it has been confirmed in pigs that proper supplementation of DL-HMTBA promoted the colonization of *Lactobacillus* in the intestinal tract (Kaewtapee et al., 2016; Malik et al., 2009). A number of studies indicated that *Lactobacillus* exhibits favorable probiotic properties which enhance epithelial barrier integrity (Wang et al., 2018; Yang et al., 2015) and inhibit inflammation (Kopp et al., 2008; Wang et al., 2016). Therefore, we speculate that the positive effects of DL-HMTBA on the intestinal barrier and inflammatory cytokine expressions could be partly attributed to the impact on the ileal microbiome. We further investigated the role of methionine source in chicken gut microbiota on different levels by *t*-test. The elevated abundance of *Blautia* in the 21 d ileum of the DL-HMTBA-high TASS level treated group was observed in our data. As a genus of anaerobic bacteria with probiotic characteristics, *Blautia* has been of particular interest since its establishment for its contribution to alleviating inflammatory diseases and metabolic diseases and for its antibacterial activity against specific microorganisms (Liu et al., 2021). On the other hand, our data also showed that DL-methionine enriched *Blautia* in the 21 d cecum when added at a low level. Taken together, we can reasonably speculate that the effect of DL-HMTBA on ileal microbiota composition was positive.

SCFA are the metabolites derived from the bacterial fermentation of dietary fibers, which link host nutrition to intestinal homeostasis maintenance. In this study, we found that SCFA such as acetate, propionate, and butyrate, were concomitantly influenced by DL-HMTBA supplementation. Numerous studies have demonstrated the beneficial impact of SCFA on host immune response and barrier functions. For example, butyrate reduces epithelial permeability by the regulation of IL-10 receptors and reinforcing TJ in vitro (Wang et al., 2012; Zheng et al., 2017). In addition, SCFA improved mucosal immune homeostasis by up-regulating anti-inflammatory cytokines and down-regulating pro-inflammatory cytokines (Maslowski et al., 2009). In line with these findings, our results of cecal microbiota composition at d 21 showed that DL-HMTBA enriched butyrate-producing bacteria such as *Intestinimonas* (Yao et al., 2020) and *Oscillospira* (Jiao et al., 2018). To our knowledge, this is the first report on DL-HMTBA that showed the beneficial effects on SCFA production in broiler chickens. Collectively, our findings confirmed the positive role of DL-HMTBA in improving broiler chickens' intestinal homeostasis.

5. Conclusions

Our data indicated that DL-HMTBA supplementation could improve the intestinal health status of broilers by improving intestinal physical barrier function and morphology, optimizing ileum microbiota, and increasing SCFA content in cecal digesta although further study on protein expression levels is warranted. The optimal TSAA level is not consistent among the intestinal health dimensions and the middle-level TSAA group in our study supported better intestinal morphology. These findings provide a new standpoint for understanding the beneficial role of DL-HMTBA in promoting intestinal homeostasis.

Author contributions

Lu Gong: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft. **Tahir Mahmood:** Conceptualization, Resources, Writing - review & editing. **Yves Mercier:** Conceptualization, Resources, Writing - review & editing. **Huiping Xu:** Methodology, Investigation. **Xiaodan Zhang:** Investigation. **Yizhu Zhao:** Methodology. **Yimeng Luo:** Methodology. **Yuming Guo:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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.

Acknowledgments

This work has been supported by Adisseo France S.A.S (202104810410901) and China Agriculture Research System Program (CA RS-41).

Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2023.07.004>.

References

- Abd EM, El-Saadony MT, Alqhtani AH, Swelum AA, Salem HM, Elbestawy AR, et al. The relationship among avian influenza, gut microbiota and chicken immunity: an updated overview. *Poult Sci* 2022;101:102021.
- Agostini PS, Dalibard P, Mercier Y, Van der Aar P, Van der Klis JD. Comparison of methionine sources around requirement levels using a methionine efficacy method in 0 to 28 day old broilers. *Poult Sci* 2016;95:560–9.
- Batonon-Alavo DI, Bouza B, Cholet JCG, Mercier Y. A method for determination of the acidifying value of organic acids used in pigs diets in the acid binding capacity at pH 4 (ABC-4) system. *Anim Feed Sci Technol* 2016;216:197–203.
- Bauchart-Thevret C, Stoll B, Chacko S, Burrin DG. Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. *Am J Physiol Endocrinol Metab* 2009;296:E1239–50.
- Beaumont M, Blachier F. Amino acids in intestinal physiology and health. *Adv Exp Med Biol* 2020;1265:1–20.
- Cakmaz R, Buyukasik O, Kahramansoy N, Erkol H, Col C, Boran C, et al. A combination of plasma DAO and citrulline levels as a potential marker for acute mesenteric ischemia. *Libyan J Med* 2013;8:20596.
- Chen Y, Li DF, Dai ZL, Piao XS, Wu ZL, Wang B, et al. L-methionine supplementation maintains the integrity and barrier function of the small-intestinal mucosa in post-weaning piglets. *Amino Acids* 2014;46:1131–42.
- Dahiya JP, Hoehler D, Van Kessel AG, Drew MD. Effect of different dietary methionine sources on intestinal microbial populations in broiler chickens. *Poult Sci* 2007;86:2358–66.
- Dang DX, Zhou HZ, Lou YJ, Li DS. Effects of methionine and/or disaccharide injected in the amnion of geese on post-hatching pectoral muscle and small intestine development, glycogen reserves, jejunum morphology, and digestive enzymes activities. *Poult Sci* 2022;101:101867.
- Fasina YO, Lillehoj HS. Characterization of intestinal immune response to *Clostridium perfringens* infection in broiler chickens. *Poult Sci* 2019;98:188–98.
- Frankel WL, Zhang W, Afonso J, Klurfeld DM, Don SH, Laitin E, et al. Glutamine enhancement of structure and function in transplanted small intestine in the rat. *JPN J Parenter Enteral Nutr* 1993;17:47–55.
- Gan LP, Fan H, Mahmood T, Guo YM. Dietary supplementation with vitamin C ameliorates the adverse effects of *Salmonella* Enteritidis-challenge in broilers by shaping intestinal microbiota. *Poult Sci* 2020;99:3663–74.
- Garcia RA, Stipanuk MH. The splanchnic organs, liver and kidney have unique roles in the metabolism of sulfur amino acids and their metabolites in rats. *J Nutr* 1992;122:1693.
- Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. *Biochim Biophys Acta* 2008;1778:729–56.
- Gottardo ET, Prokoski K, Horn D, Viott AD, Santos TC, Fernandes JL. Regeneration of the intestinal mucosa in *Eimeria* and *E. coli* challenged broilers supplemented with amino acids. *Poult Sci* 2016;95:1056–65.
- Hong YH, Lillehoj HS, Lillehoj EP, Lee SH. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria* maxima infection of chickens. *Vet Immunol Immunopathol* 2006;114:259–72.
- Ji J, Xu YB, Zheng MZ, Luo CL, Lei HT, Qu H, et al. Methionine attenuates lipopolysaccharide-induced inflammatory responses via DNA methylation in macrophages. *ACS Omega* 2019;4:2331–6.
- Jiao N, Baker SS, Nugent CA, Tsompana M, Cai L, Wang Y, et al. Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: a meta-analysis. *Physiol Genomics* 2018;50:244–54.
- Kaewtapee C, Krutthai N, Bunchasak C. Effects of supplemental liquid DL-methionine hydroxy analog free acid in diet on growth performance and gastrointestinal functions of piglets. *Asian-Australas J Anim Sci* 2016;29:1166–72.

- Kalvandi O, Sadeghi A, Karimi A. Methionine supplementation improves reproductive performance, antioxidant status, immunity and maternal antibody transmission in breeder Japanese quail under heat stress conditions. *Arch Anim Breed* 2019;62:275–86.
- Khosshbin K, Camilleri M. Effects of dietary components on intestinal permeability in health and disease. *Am J Physiol Gastrointest Liver Physiol* 2020;319:G589–608.
- Kimura M, Sawada N, Kimura H, Isomura H, Hirata K, Mori M. Comparison between the distribution of 7H6 tight junction-associated antigen and occludin during the development of chick intestine. *Cell Struct Funct* 1996;21:91–6.
- Kopp MV, Goldstein M, Dietschek A, Sofke J, Heinzmann A, Urbaneck R. *Lactobacillus GG* has in vitro effects on enhanced interleukin-10 and interferon-gamma release of mononuclear cells but no in vivo effects in supplemented mothers and their neonates. *Clin Exp Allergy* 2008;38:602–10.
- Krutthai N, Vajrabukka C, Markvichitr K, Choothesa A, Thiengham J, Sawanon S, et al. Effect of source of methionine in broken rice-soybean diet on production performance, blood chemistry, and fermentation characteristics in weaned pigs. *Czech J Anim Sci* 2015;60:123–31.
- Lei K, Li YL, Yu DY, Rajput IR, Li WF. Influence of dietary inclusion of *Bacillus licheniformis* on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. *Poult Sci* 2013;92:2389–95.
- Li H, Wan HF, Mercier Y, Zhang XL, Wu CM, Wu XQ, et al. Changes in plasma amino acid profiles, growth performance and intestinal antioxidant capacity of piglets following increased consumption of methionine as its hydroxy analogue. *Br J Nutr* 2014;112:855–67.
- Liu XM, Mao BY, Gu JY, Wu JY, Cui SM, Wang G, et al. *Blautia*-a new functional genus with potential probiotic properties? *Gut Microbes* 2021;13:1–21.
- Ma JY, Mahfuz S, Wang J, Piao XS. Effect of dietary supplementation with mixed organic acids on immune function, antioxidative characteristics, digestive enzymes activity, and intestinal health in broiler chickens. *Front Nutr* 2021;8:673316.
- Mahapatro M, Erkert L, Becker C. Cytokine-mediated crosstalk between immune cells and epithelial cells in the gut. *Cells* 2021;10:111.
- Malik K, Hoehler D, Rademacher M, Drew MD, Van Kessel AG. Apparent absorption of methionine and 2-hydroxy-4-methylthiobutanoic acid from gastrointestinal tract of conventional and gnotobiotic pigs. *Animal* 2009;3:1378–86.
- Martin CR, Osadchiv V, Kalani A, Mayer EA. The brain-gut-microbiome axis. *Cell Mol Gastroenterol Hepatol* 2018;6:133–48.
- Martín-Venegas R, Brufau MT, Guerrero-Zamora AM, Mercier Y, Geraert P, Ferrer R. The methionine precursor DL-2-hydroxy-(4-methylthio)butanoic acid protects intestinal epithelial barrier function. *Food Chem* 2013;141:1702–9.
- Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 2009;461:1282–6.
- Mastrototaro L, Sponder G, Saremi B, Aschenbach JR. Gastrointestinal methionine shuttle: priority handling of precious goods. *IUBMB Life* 2016;68:924–34.
- Matthews DR, Li H, Zhou J, Li Q, Glaser S, Francis H, et al. Methionine- and choline-deficient diet-induced nonalcoholic steatohepatitis is associated with increased intestinal inflammation. *Am J Pathol* 2021;191:1743–53.
- Melaku M, Zhong R, Han H, Wan F, Yi B, Zhang H. Butyric and citric acids and their salts in poultry nutrition: effects on gut health and intestinal microbiota. *Int J Mol Sci* 2021;22:10392.
- Montout L, Pouillet N, Bambou JC. Systematic review of the interaction between nutrition and immunity in livestock: effect of dietary supplementation with synthetic amino acids. *Animals (Basel)* 2021;11:2813.
- Nava GM, Attene-Ramos MS, Gaskins HR, Richards JD. Molecular analysis of microbial community structure in the chicken ileum following organic acid supplementation. *Vet Microbiol* 2009;137:345–53.
- Nguyen DH, Seok WJ, Kim IH. Organic acids mixture as a dietary additive for pigs-A review. *Animals (Basel)* 2020;10:952.
- Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 2014;30:3123–4.
- Pelaseyed T, Bergstrom JH, Gustafsson JK, Ermund A, Birchenough GM, Schutte A, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol Rev* 2014;260:8–20.
- Ramalingam A, Wang X, Gabelle M, Valenzano MC, Soler AP, Ko A, et al. Dietary methionine restriction improves colon tight junction barrier function and alters claudin expression pattern. *Am J Physiol Cell Physiol* 2010;299:C1028–35.
- Saito Y, Iwatsuki K, Hanyu H, Maruyama N, Aihara E, Tadaishi M, et al. Effect of essential amino acids on enteroids: methionine deprivation suppresses proliferation and affects differentiation in enteroid stem cells. *Biochem Biophys Res Commun* 2017;488:171–6.
- Simard A, Di Pietro E, Ryan AK. Gene expression pattern of Claudin-1 during chick embryogenesis. *Gene Expr Patterns* 2005;5:553–60.
- Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. *J Transl Med* 2017;15:73.
- Stipanuk MH. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu Rev Nutr* 2004;24:539–77.
- Stojanovic O, Altirriba J, Rigo D, Spiljar M, Evrard E, Roska B, et al. Dietary excess regulates absorption and surface of gut epithelium through intestinal PPAR-alpha. *Nat Commun* 2021;12:7031.
- Takiishi T, Fenero C, Camara N. Intestinal barrier and gut microbiota: shaping our immune responses throughout life. *Tissue Barriers* 2017;5:e1373208.
- Tykalowski B, Smialek M, Koncicki A, Ognik K, Zdunczyk Z, Jankowski J. The immune response of young turkeys to haemorrhagic enteritis virus infection at different levels and sources of methionine in the diet. *BMC Vet Res* 2019;15:387.
- Vazquez-Anon M, Gonzalez-Esquerria R, Saleh E, Hampton T, Ritcher S, Firman J, et al. Evidence for 2-hydroxy-4(methylthio) butanoic acid and DL-methionine having different dose responses in growing broilers. *Poult Sci* 2006;85:1409–20.
- Wang HB, Wang PY, Wang X, Wan YL, Liu YC. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. *Dig Dis Sci* 2012;57:3126–35.
- Wang J, Ji HF, Wang SX, Liu H, Zhang W, Zhang DY, et al. Probiotic *Lactobacillus plantarum* promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Front Microbiol* 2018;9:1953.
- Wang ZL, Wang L, Chen Z, Ma XY, Yang XF, Zhang J, et al. In vitro evaluation of swine-derived *Lactobacillus reuteri*: probiotic properties and effects on intestinal porcine epithelial cells challenged with enterotoxigenic *Escherichia coli* K88. *J Microbiol Biotechnol* 2016;26:1018–25.
- Wu B, Li L, Ruan T, Peng X. Effect of methionine deficiency on duodenal and jejunal IgA(+) B cell count and immunoglobulin level of broilers. *Iran J Vet Res* 2018;19:165–71.
- Wu BY, Cui HM, Peng X, Fang J, Cui W, Liu XD. Effect of methionine deficiency on the thymus and the subsets and proliferation of peripheral blood T-cell, and serum IL-2 contents in broilers. *J Integr Agric* 2012;11:1009–19.
- Wu YQ, Yin XN, Wang YL, Mahmood T, Shahid M, Yin DF, et al. Effect of 2-hydroxy-4-(methylthio) butanoic acid and acidifier on the performance, chyme pH, and microbiota of broilers. *Anim Sci J* 2020;91:e13409.
- Xu ZR, Hu CH, Xia MS, Zhan XA, Wang MQ. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poult Sci* 2003;82:1030–6.
- Yang FJ, Wang AN, Zeng XF, Hou CL, Liu H, Qiao SY. *Lactobacillus reuteri* I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. *BMC Microbiol* 2015;15:32.
- Yao Y, Yan LJ, Chen H, Wu N, Wang WB, Wang DS. Cyclocarya paliurus polysaccharides alleviate type 2 diabetic symptoms by modulating gut microbiota and short-chain fatty acids. *Phytomedicine* 2020;77:153268.
- Zhang BB, Gan LP, Shahid MS, Lv ZP, Fan H, Liu D, et al. In vivo and in vitro protective effect of arginine against intestinal inflammatory response induced by *Clostridium perfringens* in broiler chickens. *J Anim Sci Biotechnol* 2019;10:73.
- Zhang BB, Li G, Shahid MS, Gan LP, Fan H, Lv ZP, et al. Dietary l-arginine supplementation ameliorates inflammatory response and alters gut microbiota composition in broiler chickens infected with *Salmonella enterica* serovar Typhimurium. *Poult Sci* 2020;99:1862–74.
- Zhang LB, Guo YM. Effects of liquid DL-2-hydroxy-4-methylthio butanoic acid on growth performance and immune responses in broiler chickens. *Poult Sci* 2008;87:1370–6.
- Zheng L, Kelly CJ, Battista KD, Schaefer R, Lanis JM, Alexeev EE, et al. Microbial-derived butyrate promotes epithelial barrier function through IL-10 receptor-dependent repression of Claudin-2. *J Immunol* 2017;199:2976–84.