Impact of drinking water supplemented 2-hydroxy-4-methylthiobutyric acid in combination with acidifier on performance, intestinal development, and microflora in broilers

Y. J. Guo,[*,1] Z. Y. Wang,[*,1] Y. S. Wang,[*] B. Chen,[*] Y. Q. Huang,[*] P. Li,[†] Q. Tan [†],[†] H. Y. Zhang [*,2] and W. Chen[*]

[*]*College of Animal Science and Technology, Key Laboratory of Animal Biochemistry and Nutrition, Ministry of Agriculture, Henan Agricultural University, Zhengzhou, China, 450002; and* [†]*Novus International, Shanghai, China, 200080*

ABSTRACT In addition to offering methionine, 2-hydroxy-4-methylthiobutyric acid (**HMTBa**) is also an organic acid and shows excellent bacteriostasis. Therefore, 3 experiments were conducted to determine the influence of drinking water supplemented HMTBa in combination with acidifier on performance, intestinal development, and microflora in broilers. The addition of different concentration (0.02−0.20%) of the blend of HMTBa and other acids significantly reduced the pH of water and exerted antimicrobial activity in dose-dependent manner in vitro. The outcomes from animal trial consisting of the drinking water with blended acidifier at 0.00, 0.05, 0.10, 0.15, and 0.20% indicated that the water with 0.15 or 0.20% acidifier resulted in linear and quadratic higher body weight at 42 d, gain and water consumption during 1 to 42 d ($P < 0.05$). In experiment 3, responding to graded blended acidifier in drinking water, birds receiving 0.10, 0.15, and 0.20% acidifier decreased the internal pH of gastrointestinal tract and muscle, and exhibited increased duodenal weight, length, villus high, and the ratio of villus high to crypt depth. Drinking water with 0.2% blended acidifier increased the abundance of probiotics (*Bacteroidaceae, Ruminococcaceae*, and *Lachnospiraceae*) and decreased the account of pathogenic bacteria such as *Desulfovibrionaceae*. Alternations in gut microflora were closely related to the metabolism of carbohydrate, amino acid, and vitamins. These findings, therefore, suggest that drinking water with 0.10 to 0.13% the combination HMTBa with acidifier might benefit to intestinal development and gut microbiota, and the subsequent produce a positive effect on the performance of broilers.

Key words: HMTBa and acids, performance, intestinal development, broiler

2022 Poultry Science 101:101661
https://doi.org/10.1016/j.psj.2021.101661

INTRODUCTION

Combining with the global demand for safe human food and the production of environmentally friendly poultry products, acidifiers have been receiving considerable attention due to its positive effect on gut microbiota, the nutrient digestibility, and performance (Pearlin et al., 2020). Emerging evidences indicated that the diets supplemented of several organic acids exerted a favorable role in gastric proteolysis, protein and amino acids digestibility (Symeon et al., 2010), mineral absorption (Boling-Frankenbach et al., 2001), and the subsequent positive effect on the performance of broilers (Dehghani-Tafti and Jahanian, 2016). Other significant benefit related to dietary acidification is inhibiting pathogens (Roth et al., 2017), for example, diet supplemented with malic acid was found to decrease *Escherichia coli* counts in the small intestine of laying hens (Moharrery and Mahzonieh, 2005). However, it was well-known that dietary acidifier is ubiquitous in the modern farming industry, and it often was blaming for causing corrosion of processing equipment and volatilization during the granulating process (Zhu et al., 2014). In this context, providing organic acid through drinking water would be an alternative strategy for reducing these problems and inducing a positive impact on animal physiology and performance (Wales et al., 2010). For example, supplying organic acid through water was confirmed to improve the growth performance and gastrointestinal function of broilers (Aclkgoz et al., 2011). Of note, acidifiers added to drinking water was found not only to

Received July 7, 2021.
Accepted November 25, 2021.

[1]These authors contributed to this research work equally.

[2]Corresponding author: Huaiyong.zhang@ugent.be

disinfect the water itself (Krug et al., 2012), but also improve immunological parameters of birds (Aclkgoz et al., 2011; Hamed and Hassan, 2013), especially under the feed withdrawal period before slaughter. Studies showed that feed withdrawal could result in lactic acid reduction, probably as a consequence, it would increase *Salmonella* contamination, and further acidified water treatments could decrease the chance of becoming infected (Ramirez et al., 1997). These findings underline the importance of supplying acidifier in drinking water in inhibiting pathogenic bacteria and improve performance.

As a one of important methionine sources, 2-hydroxy-4-methylthiobutyric acid (**HMTBa**) is characterized by efficient absorption along the entire gastrointestinal tract of birds (Richards et al., 2005; Zou et al., 2015), and exhibited key functions in poultry, including protein synthesis, innate immune system regulation, oxidative stress defense, and modulating gut microflora (Wang et al., 2019; Rasch et al., 2020; Wu et al., 2020). More important, HMTBa also shows excellent antibacterial effects (Geraert et al., 2005) and the better performance than *DL*-methionine (Wang et al., 2019). This could be at least partly explained by the function of HMTBa as organic acid, in which an amine group is replaced by a hydroxyl group in the molecular structure of HMTBa (Wu et al., 2020). Dietary supplemental HMTBa has been shown to decreased the pH in the feed (Krutthai et al., 2015), which might be important in enhancing the protease activity, increasing protein hydrolysates, and stimulating protease secretion (O'Donnell et al., 2001). Furthermore, supplemental methionine via feed and drinking water has been shown to improve antioxidant status, anti-inflammatory response, growth performance, and wellbeing of broilers (Chen et al., 2013; Liu et al., 2019). Diet with a combination of *DL*-methionine and acidifier in broiler was noticed to decrease chyme pH, enhance digestive enzyme activities, and promoted growth of butyrate-producing bacteria such as *Faecalibacterium* (Wu et al., 2020), implying that the HMTBa or blend of HMTBa and other organic acids may exhibit synergetic role in antibacterial effects and growth performance of broilers. However, limited information is available for broilers.

The object of the current research, therefore, was to evaluate the effect of different concentration of liquid blend of HMTBa and other organic acids on antibacterial effects, growth performance, and gut microbiota of broilers. Specifically, the effects of different concentrations of liquid blended acids on the acidity and hardness of water, as well as the bacteriostasis was firstly evaluated in vitro, then 2 animal trials were carried out to define the response of the performance to blended acidifier and evaluate the impact of acidifier on intestine development and gut microflora, respectively.

MATERIALS AND METHODS

All procedures in the present study were performed in accordance with the Animal Care Committee of Henan Agricultural University (approval No. HNND20190612).

Acidifier and Preparing

In the present study, the blended acidifier includes HMTBa (ACTIVATE WD) was obtained from Novus International Co., Ltd. (Shanghai, China), the proportion of HMTBa is greater than 44% when it is added to lactic and phosphoric acid as a liquid acid blend. The work concentration in this trail is 0.00, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, and 0.20%, respectively.

Screening for Antimicrobial Activity In Vitro (Experiment 1)

The aim of this experiment was to examine the impact of different concentration of acidifier on acidity and hardness of drinking water, as well as the antibacterial effect. With this aim, the hardness of water was adjusted into 100, 200, and 400 mg/L by $CaCO_3$, and then mixed with various concentrations of acidifier including 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, or 0.20%. Subsequently, the water acidity expressed as pH value and hardness was measured using pH meter (Hanna Instruments, Inc., Woonsocket, RI) and ethylene diamine tetraacetic acid (**EDTA**) titration, respectively.

Three pathogenic test laboratory strains including *Salmonella, Staphylococcus*, and *Escherichia coli* were purchased from China Microbiological Culture Collection Center and used to analyze their sensitivity towards the acidifier. Lysogeny Broth (**LB**) agar (including tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.2-7.4.) was prepared and autoclaved at 120°C. Fresh cultures of *Salmonella, Staphylococcus*, and *Escherichia coli*, with the absorbance of 0.5 at 600 nm, were flooded onto nutrient LB agar plates. Equidistant wells (2 mm in diameter) were created in each plate to test the 2 volumes of acidifier. The plates were then incubated overnight at 37°C to determine the inhibition zone. The inhibition zones were observed and the diameter was measured with a simple ruler. The assay was performed in triplicates.

The Effect of the Combination HMTBa With Acidifier on Performance in Broilers (Experiment 2)

A total of 600 one-day-old mixed-sex Arbor Acre broilers were weighed individually and allocated to 5 treatment groups with 5 replicate pens (10 females and 10 males birds/pen) for 42 d, which consisted of the drinking water with acidifier at 0.00, 0.05, 0.10, 0.15, or 0.20%, respectively. All birds were housed in a climate-controlled facility with the initial ambient temperature set at approximately 34°C. Thereafter, the temperature was gradually reduced based on normal management practices to 22°C by 20 d. The light schedule was 23L: 1D and 18L: 6D during 1 to 7 d and beyond,

respectively. Diets included starter (1−21 d) and finisher (22−42 d) diets were formulated to meet the requirements recommended by the National Research Council (1994) and were supplied as pellets (Table 1). Of note, each cage has a separate sink, and the water consumption was recorded at 7: 00 am and 19: 00 pm every day. Body weight (**BW**), feed intake (**FI**), and mortality by pen were recorded during the trial period. Body gain and feed conversion as the feed to gain (**F: G**) was calculated on a per-pen basis.

The Effect of the Combination HMTBa With Acidifier on the Intestinal Development and Microflora of Broilers (Experiment 3)

A total of 250 one-d-old male Arbor Acre broilers were randomly divided into 5 treatment groups with 5 replicate pens (10 birds per pen) for each treatment. The five experimental groups were as follows: 1) drinking water without acidifier (Control); 2) drinking water with 0.05% acidifier; 3) drinking water with 0.10% acidifier; 4) drinking water with 0.15% acidifier; 5) drinking water with 0.20% acidifier. Dietary regimes and program of temperature, lighting, and management were identical with Experiment 2. At the age of 21 and 42 d, after fasting for 12 h, one bird of average BW from each pen was randomly selected and slaughtered by cervical dislocation. Duodenum, jejunum, and ileum were removed for length and weight determination, the mid-duodenum, mid-jejunum, and mid-ileum were dissected and rapidly immersed in phosphate-buffered formaldehyde for histology analysis. Chyme from gizzard, glandular stomach, duodenum, jejunum, ileum, and cecum, as well as pectoralis and leg muscles were obtained for pH measurement. Moreover, cecum chyme was collected and immediately frozen in the liquid nitrogen for microbial 16S rDNA sequencing.

Table 1. Composition and nutrient analysis of experimental diet (as-fed basis).

Item	1−21 d	22−42 d
Ingredients, %		
Corn	55.00	60.48
Soybean meal	32.7	25.8
Corn protein flour	5.0	5.0
Dicalcium phosphate	1.5	1.0
Stone powder	1.1	1.0
Sodium chloride	0.3	0.3
Soybean oil	3.0	5.3
Premix[1]	0.6	0.6
DL-methionine, 98%	0.21	0.16
L-lysine hydrochloride, 98.5%	0.46	0.31
L-threonine, 98.5%	0.13	0.05
Total	100	100
Nutrient analysis,[2] %		
Metabolizable energy, kcal/kg	3,000	3,202
Crude protein	23.20	19.78
Calcium	0.82	0.67
Total phosphorus	0.56	0.50
Lysine	1.44	1.15
Methionine	0.56	0.47
Threonine	0.97	0.78

[1]Premix is provided per kilogram of diet: 1−21 d: Vitamin A, 12,000 IU; Vitamin D_3, 3,500 IU; Vitamin E, 60 IU; Vitamin K_3, 4 mg; Vitamin B_1, 2.5 mg; Vitamin B_6, 6 mg; Vitamin B_{12}, 8μg; D-Pantothenic acid, 40 mg; Niacin, 75 mg; Folic acid, 10 mg; Biotin, 0.8 mg; Choline, 700 mg; Zn, 90 mg; Fe, 110 mg; Cu, 20 mg;; Mn, 100 mg; I, 0.5 mg; phytase, 0.1 g. 22−42 d: Vitamin A, 10,000 IU; Vitamin D_3, 3,000 IU; Vitamin E, 50 IU; Vitamin K_3, 3.5mg; Vitamin B_1,2 mg; Vitamin B_6, 5 mg; Vitamin B_{12}, 6μg; D-pantothenic acid, 20 mg; Niacin, 60 mg; folic acid, 8 mg; biotin, 0.6 mg; choline, 600 mg; Zn, 80 mg; Fe, 100 mg; Cu, 15 mg; Mn, 80 mg; I, 0.5 mg; phytase, 0.1 g.

[2]Metabolizable energy, methionine, lysine, and threonine in the nutritional level were calculated values, and the rest were measured values.

Intestine Histological Analysis

Formalin-fixed intestinal samples were dehydrated, embedded, sliced into 5-μm transects, and stained with hematoxylin and eosin (**H&E**), and subsequently villus height (**VH**) and crypt depth (**CD**) of at least ten well-oriented villi, were measured and the ratio of the villus height to the crypt depth (**VH/CD**) was calculated. All histomorphometry data acquisition was performed using Olympus microscope and image analysis software (Olympus, Tokyo, Japan).

Gut Microbiome

Total bacterial genomic DNA was extracted from digesta samples of cecum by use of the Stool DNA Kits. After evaluation of DNA concentration and purity, the V3−V4 hypervariable region of the bacterial 16S rRNA was amplified using the specific primer (F: 5'-CCTACGGGRSGCAGCAG-3'; R: 5'- GGACTACVVGGGTATCTAATC-3'). The 16S rDNA high-throughput sequencing was performed using the Illumina platform (Illumina, San Diego, CA). The obtained sequences were processed for alignment and cluster into OTUs at 97% similarity using USEARCH (v7.0.1090) in QIIME software. The alpha diversity was evaluated by calculating the Chao1 and Shannon index using QIIME software (Caporaso et al., 2010). Beta-diversity at genus level was estimated by calculating Bray-Curtis dissimilarity and visualized with analysis of similarities (Warton et al., 2012). Differentially enriched Kyoto Encyclopedia of Genes and Genomes (**KEGG**) functional pathways were also calculated.

Statistical Analysis

The statistical power of 0.80 (80%) was obtained in this study when the minimally detectable effect size was 1.0 and the significance level was 0.05. Data were checked for normal distribution and equal variance using the Shapiro-Wilk and Levene's tests of SAS statistical software (version 9.2, SAS Institute, Cary, NC), respectively. One-way analysis of variance (**ANOVA**) with Tukey's post hoc test for normal distribution and Kruskal-Wallis test followed by Dunn's multiple comparisons for non-normal distribution data were performed to evaluate the effect of acidifier. In addition, polynomial contrasts and the linearity of response to analyzed dietary acidifier level were examined using linear and quadratic regression. Broken-line regression analysis was

used to estimate the recommended level of acidified water supplementation using the nonlinear regression (**NLIN**) procedure of SAS (SAS Institute). Significant differences were declared at $P < 0.05$. All data were expressed as means ± standard deviation (**SD**).

RESULTS

The Combination HMTBa With Acidifier Increases the Acidity of Drinking Water

As illustrated in Table 2, regardless of the hardness, the combination HMTBa with acidifier concentration at 0.02 to 0.20% in drinking water had no significant effect on hardness, while the acidity of drinking water expressed by pH value was linearly and quadratically increased with the increasing acidifiers (both $P < 0.001$). Supplementing 0.15 and 0.20% acidifiers resulted in the lowest pH value when compared with 0.02% acidifier group ($P < 0.05$).

Bacteriostasis In Vitro

The antimicrobial activity of the combination HMTBa with acidifier against three pathogens: *Salmonella, Staphylococcus*, and *Escherichia coli* was evaluated, and the results shown that 0.10 to 0.20% acidified water linearly and quadratically increased the inhibitory diameter of *Salmonella* and *Staphylococcus* (Figures 1B and 1C; both $P < 0.01$), whereas supplementation of the combination HMTBa with acidifier did not impact the inhibitory diameter of *Escherichia coli* in this study (Figure 1D; $P > 0.05$).

Growth Performance and Drinking Water

The effects of acidified water on growth performance were shown in Table 3. The combination HMTBa with acidifier linearly and quadratically affected the BW at 42 d and gain during 1 to 42 d (both $P < 0.05$), that is, drinking water with 0.15 or 0.2% acidifier resulted in a higher BW at 42 d ($P = 0.035$) and gain during 1 to 42 d ($P = 0.038$) when compared with 0.05 or 0.10% acidifier group ($P < 0.05$), whereas no differences were found as compared with 0.00% acidifier group. There was no significant effect of dietary treatments on FI, F: G, and mortality among the 5 groups ($P > 0.05$).

The outcomes from water consumptions recorded weekly showed that the amount of drinking water linearly and quadratically increased for 1 to 42 d (both $P < 0.01$). Specifically, the birds fed drinking water with 0.15 and 0.20% acidifier exhibited the highest consumption of water in this study (Table 4).

pH of Gastrointestinal Tract and Muscle

As presented in Table 5, the experimental treatments had no significant effect on the pH of both gastrointestinal tract and muscle at 21 d. However, the pH of chyme

Table 2. Effects of different doses of the combination HMTBa with acidifier in drinking water on acidity and hardness of water (Experiment 1).

Item	Hardness of water	Levels of acidifier in drinking water							*P*-value		
		0.02%	0.04%	0.06%	0.08%	0.10%	0.15%	0.20%	ANOVA	Linear	Quadratic
Hardness (mg/L)	100	103.74 ± 5.81	110.54 ± 1.80	113.26 ± 3.12	107.14 ± 5.89	111.22 ± 5.13	111.90 ± 6.49	98.30 ± 1.36	0.270	0.304	0.056
	200	197.58 ± 5.81	207.10 ± 4.08	210.50 ± 7.20	211.18 ± 6.56	211.18 ± 7.36	194.86 ± 1.78	191.46 ± 3.60	0.086	0.074	0.014
	400	409.06 ± 1.18	407.70 ± 2.45	404.30 ± 8.27	412.46 ± 5.93	398.18 ± 2.45	407.02 ± 5.40	400.22 ± 3.40	0.401	0.210	0.466
Acidity	100	3.77 ± 0.03^{a}	3.55 ± 0.01^{b}	3.39 ± 0.01^{bc}	3.24 ± 0.08^{cd}	3.15 ± 0.01^{de}	3.02 ± 0.01^{ef}	2.86 ± 0.03^{f}	<0.001	<0.001	<0.001
	200	3.87 ± 0.04^{a}	3.55 ± 0.04^{b}	3.38 ± 0.02^{c}	3.27 ± 0.03^{cd}	3.19 ± 0.01^{d}	3.05 ± 0.02^{e}	2.93 ± 0.02^{e}	<0.001	<0.001	<0.001
	400	3.82 ± 0.02^{a}	3.58 ± 0.02^{b}	3.46 ± 0.04^{bc}	3.32 ± 0.06^{cd}	3.18 ± 0.01^{d}	3.03 ± 0.01^{e}	2.95 ± 0.02^{e}	<0.001	<0.001	<0.001

$^{a\text{-}f}$Means within a row with different superscripts are significantly different (n = 3; $P < 0.05$).

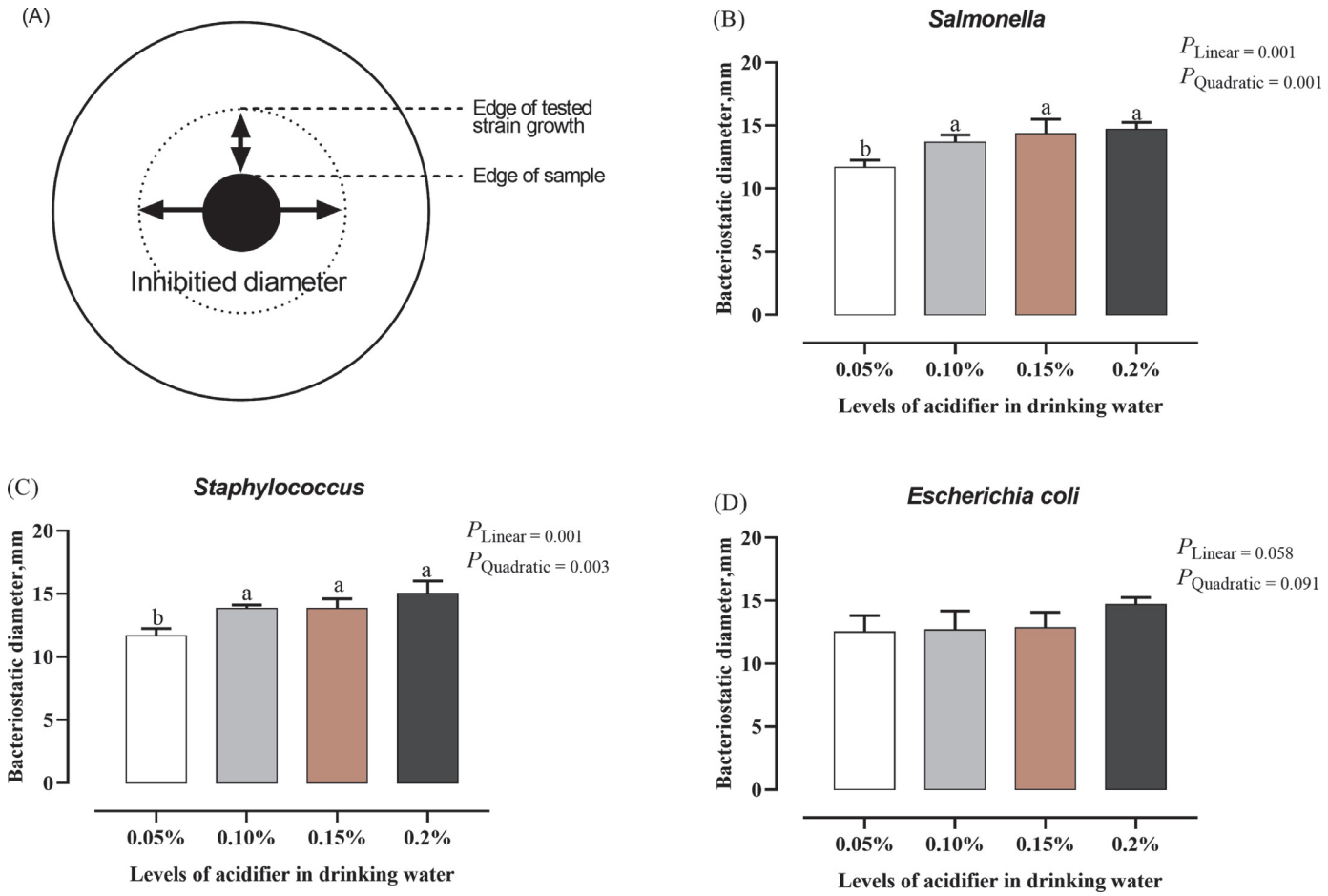

Figure 1. Bacteriostatic effect of the combination HMTBa with acidifier in drinking water (Experiment 2). (A) Schematic presentation of the antibacterial experiment. The inhibitory diameter of the combination HMTBa with acidifier against (B) *Salmonella*, (C) *Staphylococcus*, (D) and *Escherichia coli*. Values are means and standard deviation (SD) represented by vertical bars. [a,b] Mean values with different letters are significantly different (n = 3; one-way repeated measure ANOVA, $P < 0.05$, Tukey's post hoc test).

and muscle at 42 d were linearly and quadratically decreased by acidified water ($P < 0.05$). Compared with the 0.00% group, the birds fed 0.20% acidifier had notably decreased pH value in glandular stomach, duodenum, jejunum, ileum, and cecum relative at 42 d.

Intestine Development

The length and weight of small intestine at 21 and 42 d are shown in Table 6. The acidifier treatment had no significant effect on the intestinal length and

Table 3. Effect of the combination HMTBa with acidifier on the growth performance of broilers (Experiment 2).

	Levels of acidifier in drinking water					*P*-value		
Item	0.00%	0.05%	0.10%	0.15%	0.20%	ANOVA	Linear	Quadratic
BW, g/bird								
21 d	550.03 ± 64.66	514.68 ± 34.81	543.84 ± 43.12	550.08 ± 68.03	541.67 ± 45.08	0.762	0.781	0.894
42 d	2,075.44 ± 142.44[ab]	1,992.96 ± 173.37[b]	2,030.32 ± 140.89[b]	2,227.42 ± 172.83[a]	2,241.58 ± 173.81[a]	0.035	0.012	0.018
Gain, g/bird								
1−21 d	503.59 ± 26.43	468.19 ± 14.16	497.55 ± 17.70	503.48 ± 27.71	495.16 ± 18.38	0.761	0.784	0.895
21−42 d	1,525.41 ± 45.50	1,478.28 ± 57.93	1,486.49 ± 42.40	1,677.33 ± 86.28	1,699.91 ± 78.25	0.051	0.013	0.021
1−42 d	2,029.00 ± 58.16[ab]	1,946.47 ± 70.79b[b]	1,984.04 ± 57.66[b]	2,180.82 ± 70.58[a]	2,195.08 ± 71.01[a]	0.038	0.013	0.019
FI, g/bird								
1−21 d	791.21 ± 24.6	776.99 ± 21.08	828.56 ± 25.23	802.66 ± 13.7	782.09 ± 10.81	0.399	0.909	0.498
21−42 d	2,894.10 ± 75.38	2,925.00 ± 57.79	2,922.28 ± 82.94	2,958.03 ± 90	2,917.80 ± 34.48	0.980	0.709	0.861
1−42 d	3,683.08 ± 90.35	3,701.93 ± 72.04	3,756.81 ± 95.57	3,768.43 ± 98.99	3,701.95 ± 43.35	0.932	0.681	0.714
F: G, g: g								
1−21 d	1.59 ± 0.08	1.67 ± 0.08	1.67 ± 0.04	1.62 ± 0.09	1.59 ± 0.05	0.859	0.797	0.549
21−42 d	1.90 ± 0.06	2.00 ± 0.11	1.97 ± 0.06	1.79 ± 0.12	1.74 ± 0.09	0.206	0.066	0.075
1−42 d	1.82 ± 0.06	1.92 ± 0.10	1.90 ± 0.05	1.74 ± 0.07	1.70 ± 0.06	0.147	0.068	0.058
Mortality, %								
1−21 d	0.01 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.01 ± 0.01	0.535	0.753	0.346
21−42 d	0.07 ± 0.02	0.05 ± 0.03	0.07 ± 0.03	0.05 ± 0.02	0.01 ± 0.01	0.441	0.155	0.332
1−42 d	0.08 ± 0.03	0.08 ± 0.04	0.11 ± 0.05	0.08 ± 0.03	0.02 ± 0.02	0.445	0.325	0.201

[ab]Means within a row with different superscripts are significantly different (n = 6; $P < 0.05$).Abbreviations: BW, body weight; FI, feed intake; F: G, feed intake to gain ratio.

Table 4. Effect of the combination HMTBa with acidifier in drinking water on water consumption of broilers (mL/bird) (Experiment 2).

	Levels of acidifier in drinking water					*P*-value		
Item	0.00%	0.05%	0.10%	0.15%	0.20%	ANOVA	Linear	Quadratic
1−7 d	50.52 ± 0.98[ab]	47.40 ± 2.10[b]	46.93 ± 0.61[b]	51.94 ± 1.36[ab]	55.45 ± 2.04[a]	0.003	0.015	<0.001
7−14 d	63.87 ± 0.67[bc]	60.34 ± 1.27[c]	64.63 ± 0.87[b]	67.33 ± 1.40[ab]	69.24 ± 0.64[a]	<0.001	<0.001	<0.001
14−21 d	116.81 ± 1.21	112.97 ± 3.54	117.22 ± 4.18	118.89 ± 2.14	119.80 ± 2.57	0.531	0.197	0.359
1−21 d	77.21 ± 0.60[bc]	73.33 ± 1.01[c]	76.33 ± 2.26[bc]	80.39 ± 1.51[ba]	82.73 ± 1.13[a]	0.001	0.003	0.001
21−28 d	169.36 ± 3.22	162.00 ± 3.35	171.85 ± 4.16	177.97 ± 7.47	178.89 ± 3.21	0.091	0.023	0.060
28−35 d	272.81 ± 7.25[b]	271.51 ± 6.29[b]	295.47 ± 9.95[ab]	312.67 ± 7.92[a]	319.64 ± 9.70[a]	0.001	<0.001	<0.001
35−42 d	287.63 ± 4.47[b]	286.09 ± 5.25[b]	291.51 ± 13.86[b]	326.09 ± 6.67[ab]	341.29 ± 11.36[a]	0.002	<0.001	<0.001
21−42 d	252.78 ± 4.73[b]	247.00 ± 4.01[ab]	259.64 ± 12.19[ab]	276.04 ± 5.46[ab]	281.17 ± 7.63[a]	0.027	<0.001	0.004
1−42 d	157.42 ± 3.07[c]	156.07 ± 2.26[c]	163.88 ± 4.48[bc]	175.06 ± 4.30[ab]	175.06 ± 4.30[a]	<0.001	<0.001	<0.001

[abc]Means within a row with different superscripts are significantly different (n = 6; $P < 0.05$).

Table 5. Effect of the combination HMTBa with acidifier on the pH of gastrointestinal tract and muscle of broilers (Experiment 3).

		Levels of acidifier in drinking water					*P*-value		
Item		0.00%	0.05%	0.10%	0.15%	0.20%	ANOVA	Linear	Quadratic
21 d	Gizzard	3.79 ± 0.21	3.61 ± 0.38	3.93 ± 0.52	3.09 ± 0.14	4.20 ± 0.49	0.343	0.817	0.619
	Glandular stomach	4.49 ± 0.66	4.34 ± 0.38	4.06 ± 0.35	4.47 ± 0.53	4.78 ± 0.47	0.886	0.634	0.596
	Pectoralis	6.44 ± 0.09	6.39 ± 0.11	6.45 ± 0.10	6.42 ± 0.13	6.44 ± 0.04	0.994	0.936	0.994
	Leg muscles	6.62 ± 0.07	6.65 ± 0.13	7.02 ± 0.23	6.68 ± 0.20	6.94 ± 0.10	0.285	0.194	0.403
	Duodenum	6.62 ± 0.07	6.65 ± 0.13	7.02 ± 0.23	6.68 ± 0.20	6.94 ± 0.10	0.404	0.056	0.162
	Jejunum	5.98 ± 0.53	6.68 ± 0.46	6.22 ± 0.50	6.87 ± 0.39	6.48 ± 0.24	0.635	0.387	0.563
	Ileum	6.00 ± 0.55	6.53 ± 0.34	6.27 ± 0.28	6.71 ± 0.36	6.45 ± 0.55	0.808	0.415	0.617
	Cecum	6.05 ± 0.46	6.63 ± 0.30	6.59 ± 0.27	7.01 ± 0.22	6.29 ± 0.41	0.367	0.443	0.189
42d	Gizzard	3.17 ± 0.21	3.55 ± 0.25	4.33 ± 0.75	3.13 ± 0.20	3.08 ± 0.20	0.162	0.661	0.171
	Glandular stomach	4.43 ± 0.28[a]	4.09 ± 0.24[ab]	3.77 ± 0.36[ab]	3.67 ± 0.07[ab]	3.17 ± 0.10[b]	0.015	<0.001	0.002
	Pectoralis	6.61 ± 0.08	6.48 ± 0.09	6.77 ± 0.05	6.54 ± 0.12	6.61 ± 0.05	0.183	0.827	0.905
	Leg muscles	6.59 ± 0.05	6.55 ± 0.12	6.71 ± 0.05	6.78 ± 0.12	6.65 ± 0.10	0.428	0.229	0.370
	Duodenum	6.38 ± 0.07[a]	6.31 ± 0.08[ab]	6.38 ± 0.09[ab]	5.97 ± 0.19[bc]	5.86 ± 0.20[c]	0.037	0.004	0.010
	Jejunum	6.45 ± 0.06[a]	6.33 ± 0.06[ab]	6.29 ± 0.06[ab]	6.08 ± 0.18[ab]	5.93 ± 0.17[b]	0.038	0.001	0.006
	Ileum	7.35 ± 0.08[a]	7.12 ± 0.14[ab]	6.94 ± 0.16[ab]	6.84 ± 0.13[ab]	6.69 ± 0.13[b]	0.015	<0.001	0.002
	Cecum	7.37 ± 0.38[a]	6.81 ± 0.21[ab]	6.62 ± 0.12[ab]	6.73 ± 0.09[ab]	6.42 ± 0.07[b]	0.045	0.006	0.014

[abc]Means within a row with different superscripts are significantly different (n = 5, $P < 0.05$).

weight at 21 d ($P > 0.05$). At the age of 42 d, in comparison to control group, the length of duodenum and ileum increased significantly ($P < 0.05$) by drinking water with 0.15% acidifier. Meanwhile, the acidifier in drink water had linearly and quadratically effect on duodenal and ileal weight (both $P < 0.05$). The higher weight of duodenum and ileum was observed in the level of 0.15% and 0.15−0.20% acidifier for 42-day-old broiler, respectively.

As showed in Figure 2. Acidifier concentration at 0.00-0.20% in drink water had a linearly and quadratically effect on the duodenal VH and the VH/CD ratio at 21 d, as well as the duodenal VH at 42 d. At 21 d of age, drink water with 0.20% acidifier fed birds had a higher duodenal VH and the VH/CD ratio compared to the 0.00, 0.05, or 0.10% group. Supplementation of 0.10 to 0.20% acidifier to drink water increased the duodenal VH when compared with the 0.00 or 0.05% acidifier at 42 d ($P < 0.05$).

Table 6. Effect of the combination HMTBa with acidifier on intestinal length and weight of broilers (Experiment 3).

		Levels of acidifier in drinking water					*P*-value		
Item		0.00%	0.05%	0.10%	0.15%	0.20%	ANOVA	Linear	Quadratic
21d	Duodenal length, cm	27.60 ± 1.50	29.00 ± 1.18	29.06 ± 1.63	29.38 ± 2.14	25.36 ± 3.14	0.619	0.523	0.302
	Duodenal weight, g	8.48 ± 0.47	8.66 ± 0.82	8.44 ± 0.42	8.84 ± 0.48	8.48 ± 0.87	0.991	0.925	0.976
	Jejunal length, cm	61.14 ± 2.56	56.40 ± 4.31	59.32 ± 3.55	59.10 ± 1.92	63.96 ± 3.92	0.609	0.433	0.320
	Jejunal weight, g	13.62 ± 0.96	13.84 ± 1.16	14.26 ± 0.94	14.94 ± 1.13	13.64 ± 1.33	0.907	0.737	0.746
	Ileal length, cm	64.82 ± 3.37	54.40 ± 4.39	61.06 ± 3.16	56.32 ± 2.72	60.98 ± 3.03	0.237	0.616	0.331
	Ileal weight, g	11.72 ± 0.60	11.70 ± 1.54	10.66 ± 0.56	11.64 ± 0.82	11.50 ± 1.02	0.930	0.866	0.870
42d	Duodenal length, cm	36.34 ± 1.38	33.26 ± 1.13	36.76 ± 2.58	36.78 ± 2.08	33.6 ± 0.60	0.396	0.726	0.808
	Duodenal weight, g	16.64 ± 1.42[a]	20.69 ± 1.13[ab]	20.78 ± 1.58[ab]	23.44 ± 2.22[b]	21.44 ± 044[ab]	0.052	0.006	0.012
	Jejunal length, cm	84.44 ± 8.33	66.74 ± 2.45	73.5 ± 2.93	82.32 ± 5.40	69.36 ± 4.77	0.100	0.433	0.645
	Jejunal weight, g	33.03 ± 2.81	30.88 ± 1.06	30.88 ± 1.64	34.29 ± 3.96	34.48 ± 3.14	0.794	0.454	0.545
	Ileal length, cm	71.02 ± 8.37	65.84 ± 3.94	76.46 ± 3.98	87.88 ± 4.42	76.34 ± 4.84	0.092	0.081	0.195
	Ileal weight, g	21.84 ± 0.71[a]	23.47 ± 1.03[ab]	24.02 ± 3.11[ab]	29.63 ± 2.69[b]	29.62 ± 0.82[b]	0.037	0.002	0.010

[ab]Means within a row with different superscripts are significantly different (n = 5; $P < 0.05$).

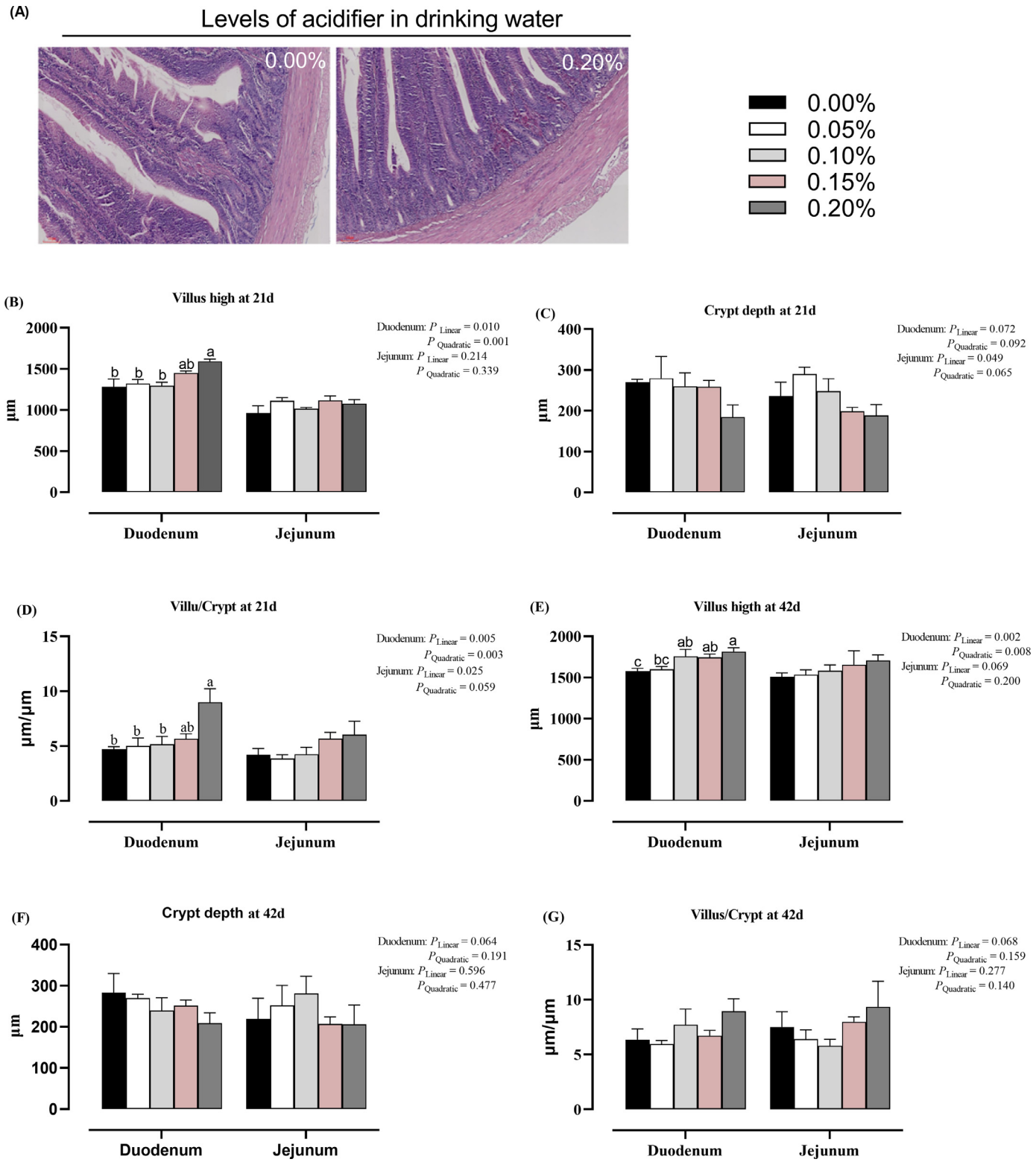

Figure 2. The combination HMTBa with acidifier supplementation improves intestinal development (Experiment 3). (A) Representative hematoxylin/eosin (H&E) staining of duodenum and (B−G) villus height and crypt depth were measured in duodenum and jejunum. Scale bar = 100μm. Values are means and standard deviation (SD) represented by vertical bars. [a-b] Mean values with different letters are significantly different (n = 5; one-way repeated measure ANOVA, $P < 0.05$, Tukey's post hoc test).

Microflora of Cecum

Figure 3 described the effects of acidifier on cecal microbiome for birds. The Chao1 and Shannon index showing alpha diversity were not notably changed by experiment treatment (Figures 3A and 3B; $P > 0.05$). According to the Figure 3C, the distances to 0.00% group was significantly different in terms of 0.2% but not 0.05% acidifier with HMTBa group, indicated that microflora in the 0.20% group formed a distinct cluster from those in the 0.00 and 0.05% group. The compositions of cecal microbiota at family level differed among groups

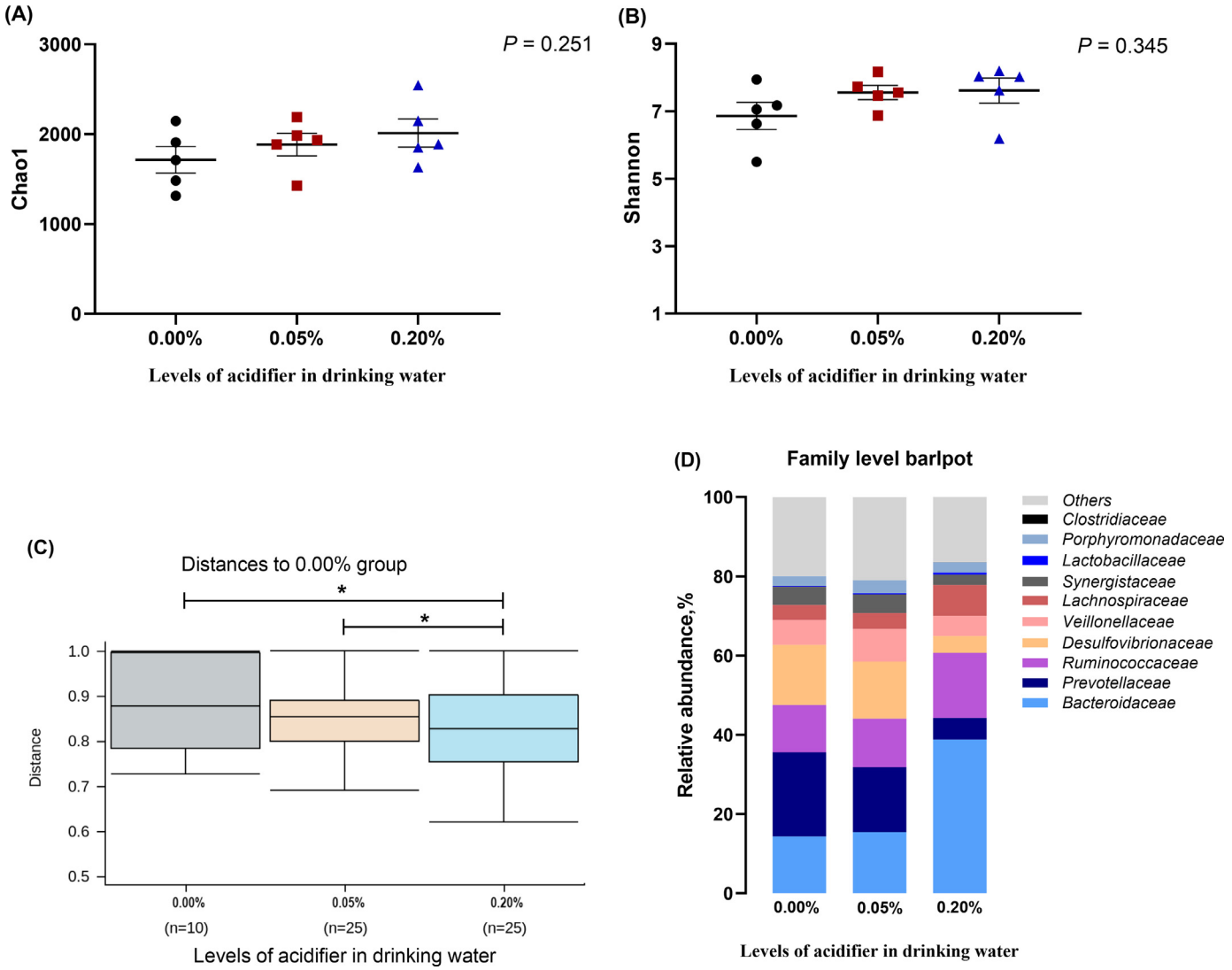

Figure 3. Effect of the combination HMTBa with acidifier on caecal microbiome in broilers at d 42 (Experiment 3). (A, B) Chao1 and Simpson indexes were used to assess diversity and evenness at genus level, (C) the distance to 0.00% group of cecum microbiome diversity at species level based on Bray-Curtis dissimilarities; (D) relative abundances of bacterial communities at family level. * denotes significant difference at $P < 0.05$.

(Figure 3D). The caecal microbiota in broilers was dominated by the *Bacteroidaceae, Prevotellaceae, Ruminococcaceae*, and *Desulfovibrionaceae*. Specifically, conjunctive using the combination of HMTBa with acidifier in drinking water distinctly increased the abundant of *Bacteroidaceae, Ruminococcaceae*, and *Lachnospiraceae*, as well as decreased the proportion of pathogenic bacteria such as *Desulfovibrionaceae* (Figure 3D).

Furthermore, the predicted KEGG metabolic pathway differences indicated that the main metabolic pathways of the functional genes in the microbial community were significantly influenced by the supplementation of the combination of HMTBa with acidifier (Figure 4). In detail, acidified water was closely related to the biosynthesis of amino acid, carbohydrate, vitamins, and nucleotide. Drinking water supplemented the combination of HMTBa with acidifier also associated with the degradation of carbohydrate and nucleotide, fermentation, and glycolysis, etc.

The Recommended Level of the Combination of HMTBa With Acidifier in Drinking Water Based on Broken-Line Analysis

The recommended level of the combination HMTBa with acidifier in drinking water using one-slope broken-line analysis are shown in Figure 5. From the perspective of BW, the weight of duodenum and ileum, and duodenal VH of 42-day-old broiler were increased with the supplementation HMTBa in combination with acidifier in drinking water ($P < 0.05$), where the recommended level of acidifier in drinking water based on BW was 0.128% (Figure 5A). As far as intestinal characteristics are concerned, the broken-line analysis revealed that the recommended level of acidifier in drinking water were 0.095, 0.110, and 0.097% based on duodenal weight, ileal weight, and VH of duodenum, respectively (Figures 5B–5D).

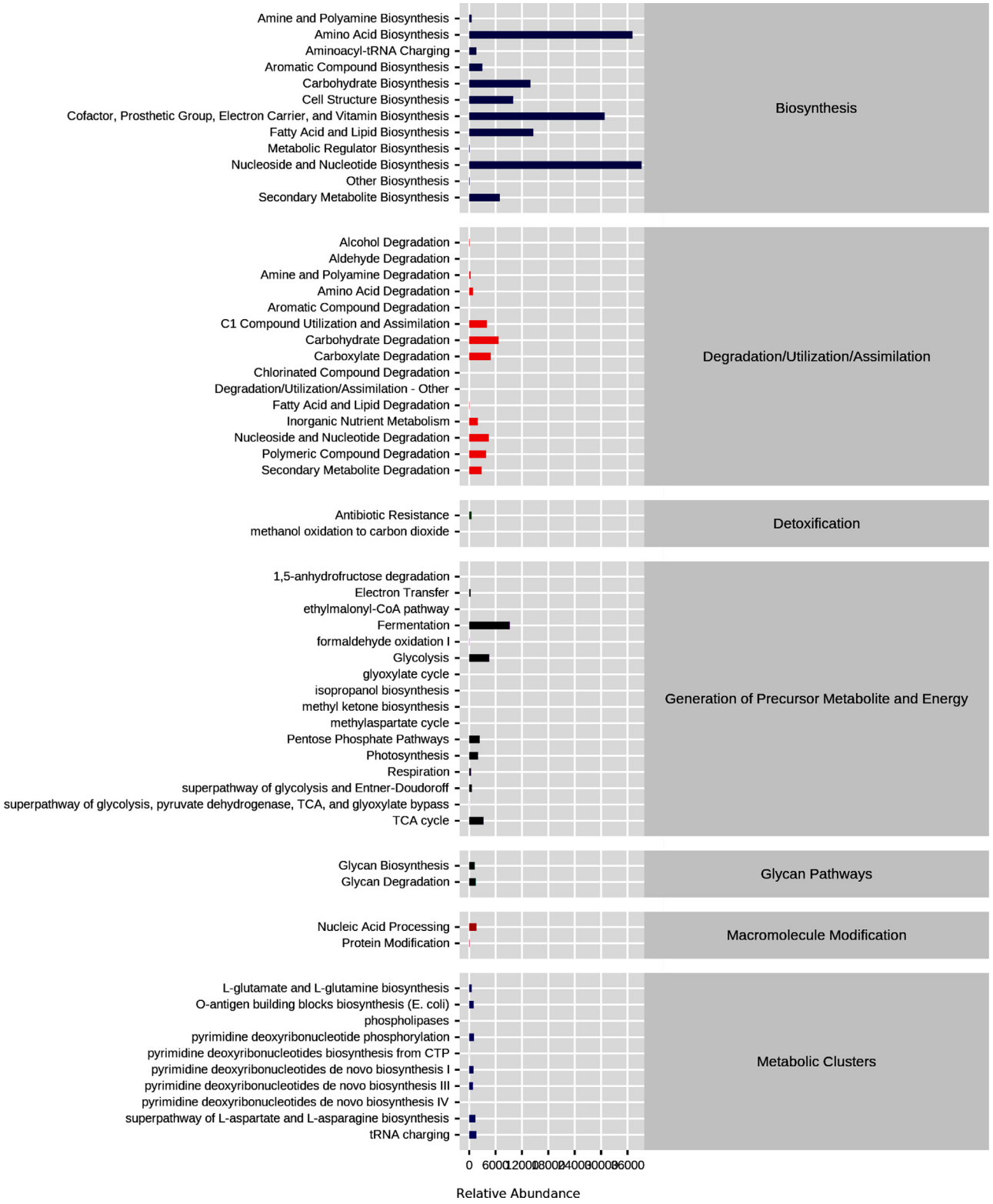

Figure 4. Functional predictions for the cecal microbiome based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

DISCUSSION

In addition to having a beneficial effect on protein synthesis, performance, and gut microflora (Wang et al., 2019; Rasch et al., 2020; Wu et al., 2020), HMTBa is also considered to exert positive role in performance as an organic acid because it has a hydroxyl group instead of an amine group (Geraert et al., 2005; Wang et al., 2019; Wu et al., 2020). In the current study, we showed that supplementation of the combination of HMTBa and acidifier to drinking water associated with improved antibacterial effects, performance, and intestinal microbiome. The specificity of the effects of the acidifier on intestinal development was demonstrated by heavier weight, increased VH and the VH/CD ratio. Besides, conjunctive using the combination of HMTBa with

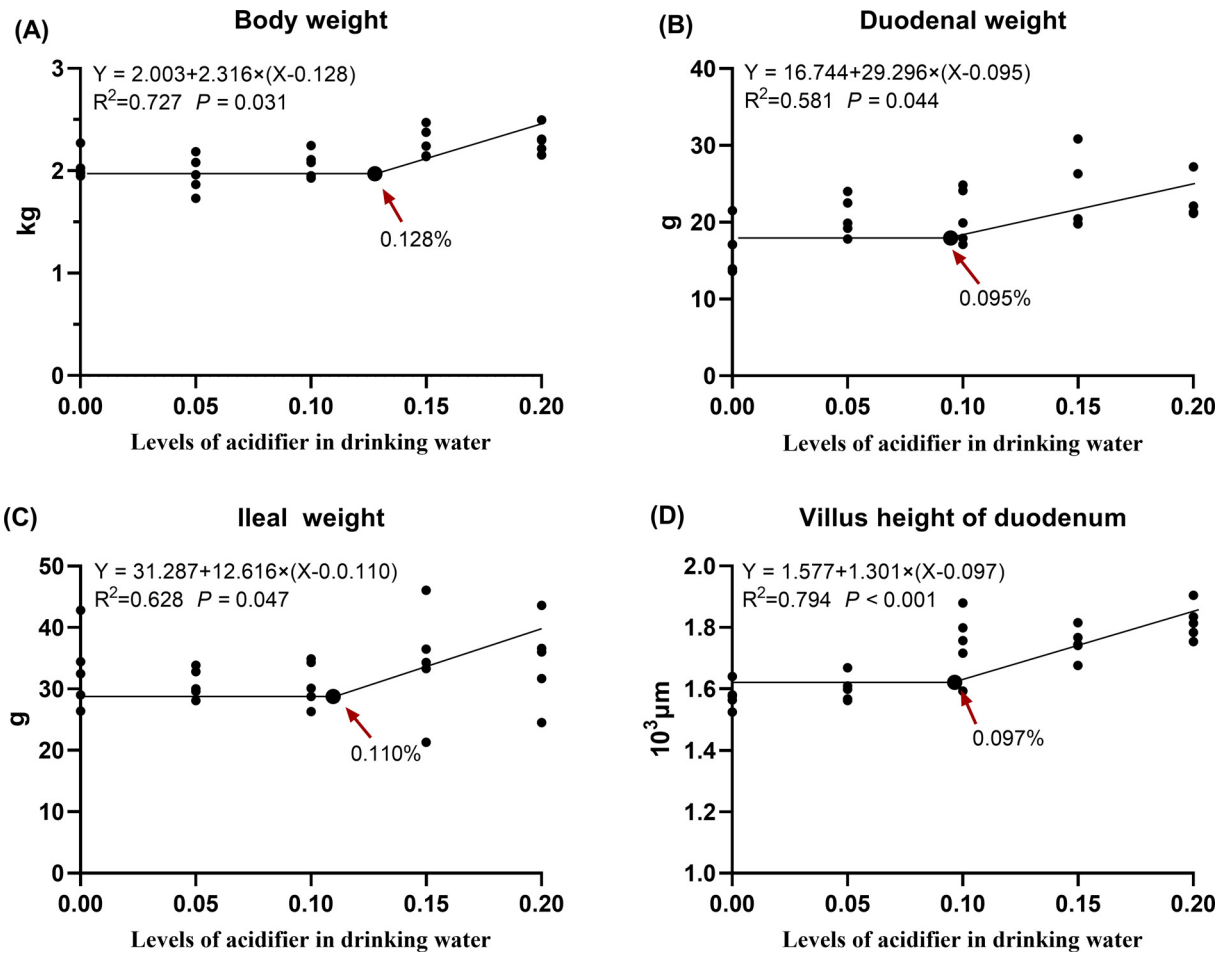

Figure 5. The recommended level of the combination of HMTBa with acidifier in drinking water based on broken-line analysis of (A) body weight (BW), (B) duodenal weight, (C) jejunal weight, and (D) villus height of duodenum.

acidifier was also noticed to improve gut microbiome such as increasing the abundance of *Bacteroidaceae, Ruminococcaceae*, and *Lachnospiraceae* along with decreasing *Desulfovibrionaceae* proportion.

Considerable research has demonstrated that organic acids in poultry diets can improve poultry performance due to partly increasing gastric proteolysis, protein, and amino acids digestibility (Dehghani-Tafti and Jahanian, 2016; Boling-Frankenbach et al., 2001; Symeon et al., 2010). Of note, organic acid supplementation in the drinking water was confirmed to improve the growth performance and gastrointestinal function in broilers (Aclkgoz et al., 2011), as well as the production performance, egg quality, and immune system in layers (Abbas et al., 2013). In line with previous reports, the data of growth performance in the current study indicated that acidification of drinking has a beneficial effect on BW and gain of broilers. Notably, diet with higher HMTBa supplementation levels (0.20 vs. 0.05%) was noticed to improve the BW, gain, and FI of 21-day-old broilers (Wang et al., 2019). Beyond our expectations, there were no significant differences in BW and gain among the 0.00, 0.15, and 0.20% acidifier groups, which may be due to the short feeding period and other mechanism. Further study is necessary to illustrate these possibilities. The increased water consumptions implied the higher intake of HMTBa in broiler during 1 to 42 d and could partly explain the improvement of growth performance by acidified drinking water. In addition, the superior growth performance could be also attributed to lower pH in digestive tract, well-developed intestine, and higher absorption capacity (Pearlin et al., 2020).

Decreased pH in digestive tract is considered as one of crucial factors impacting absorption capacity, and usually associated with an increase in gastric enzyme activity (O'Donnell et al., 2001). Some studies found that organic acids significantly reduced the pH of all gastrointestinal tract segments in broilers (Pesti et al., 2004), which was further verified by our finding that the acidified water decreased the pH value in glandular stomach and intestinal tract of broilers at 42-day-old birds but not at 21 d of age. While the pH reduction due to acidifier treatment was also deemed that mainly happened in the upper gut such as crop but not cecum (Byrd et al., 2001), indicating it is possible that the role of acidifier in pH regulation differ from tract segments, age, and their combine. It is worth stressing that decreased pH in gastrointestinal tract might not be attributed to supplemental HMTBa, although it has been shown to decrease the pH in the feed (Krutthai et al., 2015). Wu and colleague found that increased dietary HMTBa minimal effect on pH in the crop, gizzard, jejunum, and ileum in broilers (Wu et al., 2020).

Facilitating intestinal development probably is also an important contributor to the positive effects of acidifier on growth and feed efficiency. The use of organic acids has been reported to have favorable influence on jejunal morphology (Ragaa and Korany, 2016). The findings of a previous study also revealed that the VH and the VH/CD ratio of jejunum were greater in broilers that received acidified drinking water than those with normal drinking water (Eftekhari et al., 2015). In the current study, the addition of acidifier did not change the morphometric indices of jejunum significantly, whereas drinking water with 0.15 to 0.20% acidifier increased the duodenal VH and the VH/CD ratio in 21-day-old chickens and exhibited slightly increase of duodenal VH in 42-day-old birds. It was explained by reduction of the presence of toxins that are related with alterations in gut morphology of broiler chickens owe to improved microbial load (Garcia et al., 2007). Besides, drinking water supplemented HMTBa in combination with acidifier promoted intestinal development in this study might result from a better antioxidant defense mechanism. Previous research has shown that HMTBa tends to metabolize in the sulfur pathway to improve the antioxidant capacity of body (Martínez et al., 2017).

It is established that the gastrointestinal microbiota plays important roles in nutrition, immunity, and physiological systems of the chickens. For instance, *Salmonella* enhance infections and decline the growth performance of broilers (Zhang et al., 2020). An in vitro evaluation of acidifier on antimicrobial activity in this study revealed that it had a broad bactericidal activity against *Staphylococcus* and *Salmonella.* These results are consistent with previous studies saying that a significant reduction in the viability of *Salmonella* for the dietary formic acid in vitro (Al-Natour and Alshawabkeh, 2005), and reduced counts of *Staphylococcus* and *Escherichia coli* by acidified sodium chlorite in beef briskets (Hajmeer et al., 2004). To further define the relationship between supplemented HMTBa in combination with acidifier and gut microbiota, and these results showed that drinking acidifier supplementation stimulated the relative abundances of *Bacteroidaceae, Ruminococcaceae*, and *Lachnospiraceae*, these are responsible for fermentation of indigestible polysaccharides to produce short chain fatty acids which can be utilized by the host's epithelial cells. *Bacteroides*, which is a dominant flora in the human gut, plays an important role in polysaccharide metabolism (El Kaoutari et al., 2013). Metagenomics studies indicated that cecal Bacteroidetes also polysaccharide utilization systems associated with polysaccharide utilization systems in broilers (Sergeant et al., 2014). Analogously, both *Lachnospiraceae* and *Ruminococcaceae* are related to carbohydrate metabolism, that is, *Ruminococcaceae* have higher numbers of cellulase and xylanase genes for fermenting of substrates, and *Lachnospiraceae* were associated with cleavage of α-amylase bonds present in starch and glycogen (Biddle et al., 2013). In addition, members of the *Lachnospiraceae* family have also been shown to utilize mucin glycans as a sole carbon source, producing propanol and propionate (Crost et al., 2013), suggesting that it is likely that these strains possess enzymes which allow them to utilize host mucins as an energy source, especially for broilers whose had the preference *Lachnospiraceae* to reside in the mucus. The outcomes of the current study further demonstrated that the changes of gut microbiota were closely related to the carbohydrate, biosynthesis and degradation. Furthermore, conjunctive using HMTBa and acidifier was associated with amino acid biosynthesis, vitamin biosynthesis, nucleoside and nucleotide biosynthesis and degradation, etc. Of note, we detected the lower presence of *Desulfovibrionaceae* and *Prevotellaceae* in caecum of broilers due to the supplemented HMTBa in combination with acidifier. *Desulfovibrionaceae*, one of the main intestinal pathogenic bacteria producing endotoxin, has been found to induce the expression of inflammatory factors in the intestinal epithelium (Ley et al., 2006). Lower abundance of *Desulfovibrionaceae* in the present study suggested the favorable role of the combination acidifier with HMTBa in intestinal barrier. However, beyond the expected scope, decreased the portion of *Prevotellaceae* by acidified water is conflicting with the protective effect of acidifier in intestinal health. Because *Prevotellaceae* is considered to be a kind of probiotics, and not only resists the colonization of pathogenic bacteria in the intestinal epithelium, but also improves the intestinal mucosal barrier function by promoting the immune response of secretory immunoglobulin A (Song et al., 2014). In addition, high-dose HMTBa (0.284%) was also enhanced relative abundances of *Actinobacteria, Erysipelotrichaceae*, and *Clostridium* in broilers (Wu et al., 2020). Taken together, alternations of microbial structure and metabolism could be one of the possible reasons why the combination HMTBa with acidifier supplementation can promote growth performance of broilers, whereas the bidirectional regulations between microbes and acidifier should be further investigated.

There are two limitations in our study. One is the limitation of statistical assessment. Unequal variances and non-normal distribution were observed in some data sets, which contribute to an insufficient number of samples. The other one is the method of recording water consumption. Due to our insufficient skills for the affordable sensors and open-source algorithm analysis and the use of manual record, it is inevitable the effect of behaviors on the overall health status of the chickens, the AI-enabled quantification of the drinking behavior through sensor monitoring should be used to minimize these adverse effects. Therefore, these limitations probably lead to our conclusions may overestimate or underestimate the role of acidulants in performance and/or intestinal development.

In summary, drinking water supplemented of 0.10 to 0.13% the combination HMTBa with acidifier worked might change the internal pH, benefit to intestinal development and gut microbiota, and the subsequent decline in pathogenic microorganism growth might produce a positive effect on the performance of broilers.

ACKNOWLEDGMENTS

Financial support was provided by National Natural Science Foundation of China (No. 32072748), and Innovative Leading Talents Project of Zhengzhou (201845). The authors gratefully thank all the people involved in this work, and Novus International for professional advises and improving manuscript.

DISCLOSURES

The authors declare that there is no conflict of interest, financial or otherwise.

REFERENCES

Abbas, G., S. H. Khan, and H. U. Rehman. 2013. Effects of formic acid administration in the drinking water on production performance, egg quality and immune system in layers during hot season. Avian. Biol. Res. 6:227–232.

Aclkgoz, Z., H. Bayraktar, Z. Aclkgoz, H. Bayraktar, and O. Altan. 2011. Effects of formic acid administration in the drinking water on performance, intestinal microflora and carcass contamination in male broilers under high ambient temperature. Anim. Feed Sci. Technol. 24:87–94.

Al-Natour, M. Q., and K. M. Alshawabkeh. 2005. Using varying levels of formic acid to limit growth of salmonella gallinarum in contaminated broiler feed. Asian. Austral. J. Anim. 18:390–395.

Biddle, A., L. Stewart, J. Blanchard, and S. Leschine. 2013. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity. 5:627–640.

Boling-Frankenbach, S. D., J. L. Snow, C. M. Parsons, and D. H. Baker. 2001. The effect of citric acid on the calcium and phosphorus requirements of chicks fed corn-soybean meal diets. Poult. Sci. 80:783–788.

Byrd, J. A., B. M. Hargis, D. J. Caldwell, R. H. Bailey, K. L. Herron, J. L. McReynolds, R. L. Brewer, R. C. Anderson, K. M. Bischoff, T. R. Callaway, and L. F. Kubena. 2001. Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on Salmonella and Campylobacter contamination of broilers. Poult. Sci. 80:278–283.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7:335–336.

Chen, Y., X. Chen, H. Zhang, and Y. Zhou. 2013. Effects of dietary concentrations of methionine on growth performance and oxidative status of broiler chickens with different hatching weight. Br. Poult. Sci. 54:531–537.

Crost, E. H., L. E. Tailford, G. Le Gall, M. Fons, B. Henrissat, and N. Juge. 2013. Utilisation of mucin glycans by the human gut symbiont Ruminococcus gnavus is strain-dependent. PloS One. 8: e76341.

Dehghani-Tafti, N., and R. Jahanian. 2016. Effect of supplemental organic acids on performance, carcass characteristics, and serum biochemical metabolites in broilers fed diets containing different crude protein levels. Anim. Feed Sci. Tech 211:109–116.

Eftekhari, A., V. Rezaeipour, and R. Abdullahpour. 2015. Effects of acidified drinking water on performance, carcass, immune response, jejunum morphology, and microbiota activity of broiler chickens fed diets containing graded levels of threonine. Livest. Sci. 180:158–163.

El Kaoutari, A., F. Armougom, J. I. Gordon, D. Raoult, and B. Henrissat. 2013. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. Nat. Rev. Microbiol. 11:497–504.

Garcia, V., P. Catala-Gregori, F. Hernandez, M. Megias, and J. Madrid. 2007. Effect of formic acid and plant extracts on growth, nutrient digestibility, intestine mucosa morphology, and meat yield of broilers. J. Appl. Poult. Res. 16:555–562.

Geraert, P., P. Graulet, Y. Mercier, P. Becker, and van der Klis J. 2005. The bactericidal effect of methionine hydroxy analog. Pages 246−247 in Proceedings of the 17th Australian Poultry Science Symposium Poultry Research Foundation. South Wales, Australia.

Hajmeer, M. N., J. L. Marsden, D. Y. Fung, and G. K. Kemp. 2004. Water, sodium chloride and acidified sodium chlorite effects on Escherichia coli O157:H7 and Staphylococcus aureus on beef briskets. Meat Sci. 68:277–283.

Hamed, D., and A. Hassan. 2013. Acids supplementation to drinking water and their effects on Japanese quails experimentally challenged with Salmonella enteritidis. Res. Zoo. 3:15–22.

Krug, P. W., C. R. Larson, A. C. Eslami, and L. L. Rodriguez. 2012. Disinfection of foot-and-mouth disease and African swine fever viruses with citric acid and sodium hypochlorite on birch wood carriers. Vet. Microbiol. 156:96–101.

Krutthai, N., C. Vajrabukka, K. Markvichitr, A. Choothesa, J. Thiengtham, S. Sawanon, C. Kaewtapee, and C. Bunchasak. 2015. 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. 60:123–131.

Ley, R. E., P. J. Turnbaugh, S. Klein, and J. I. Gordon. 2006. Human gut microbes associated with obesity. Nature. 444:1022–1023.

Liu, G., A. Magnuson, T. Sun, S. Tolba, C. Starkey, R. Whelan, and X. Lei. 2019. Supplemental methionine exerted chemical form-dependent effects on antioxidant status, inflammation-related gene expression, and fatty acid profiles of broiler chicks raised at high ambient temperature. J. Anim. Sci. 97:4883–4894.

Martínez, Y., X. Li, G. Liu, P. Bin, W. Yan, D. Más, M. Valdivié, C. A. Hu, W. Ren, and Y. Yin. 2017. The role of methionine on metabolism, oxidative stress, and diseases. Amino Acids. 49:2091–2098.

Moharrery, A., and M. Mahzonieh. 2005. Effect of malic acid on visceral characteristics and coliform counts in small intestine in the broiler and layer chickens. Int. J. Poult. Sci. 4:761–764.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academies Press, Washington, DC.

O'Donnell, D., L. Wang, J. Xu, D. Ridgway, T. Gu, and M. Moo-Young. 2001. Enhanced heterologous protein production in Aspergillus niger through pH control of extracellular protease activity. Biochem. Eng. J 8:187–193.

Pearlin, B. V., S. Muthuvel, P. Govidasamy, M. Villavan, M. Alagawany, M. Ragab Farag, K. Dhama, and M. Gopi. 2020. Role of acidifiers in livestock nutrition and health: a review. J. Anim. Physiol. Anim. Nutr. (Berl) 104:558–569.

Pesti, G., R. Bakalli, P. Vendrell, and H. Y. Chen. 2004. Effects of organic acid on control of bacteria growth in drinking water for broilers. Poult. Sci. 83:M303.

Ragaa, N. M., and R. M. S. Korany. 2016. Studying the effect of formic acid and potassium diformate on performance, immunity and gut health of broiler chickens. Anim. Nutr. 2:296–302.

Ramirez, G. A., L. L. Sarlin, D. J. Caldwell, C. R. Yezak Jr., M. E. Hume, D. E. Corrier, J. R. Deloach, and B. M. Hargis. 1997. Effect of feed withdrawal on the incidence of Salmonella in the crops and ceca of market age broiler chickens. Poult. Sci. 76:654–656.

Rasch, I., S. Görs, A. Tuchscherer, T. Viergutz, C. C. Metges, and B. Kuhla. 2020. Substitution of dietary sulfur amino acids by dl-2-Hydroxy-4-Methylthiobutyric acid reduces fractional glutathione synthesis in weaned piglets. J. Nutr. 150:722–729.

Richards, J. D., C. A. Atwell, M. Vázquez-Añón, and J. J. Dibner. 2005. Comparative in vitro and in vivo absorption of 2-hydroxy-4(methylthio) butanoic acid and methionine in the broiler chicken. Poult. Sci. 84:1397–1405.

Roth, N., S. Mayrhofer, M. Gierus, C. Weingut, C. Schwarz, B. Doupovec, R. Berrios, and K. J. Domig. 2017. Effect of an organic acids based feed additive and enrofloxacin on the prevalence of antibiotic-resistant *E. coli* in cecum of broilers. Poult. Sci. 96:4053–4060.

Sergeant, M. J., C. Constantinidou, T. A. Cogan, M. R. Bedford, C. W. Penn, and M. J. Pallen. 2014. Extensive microbial and functional diversity within the chicken cecal microbiome. PloS One. 9: e91941.

Song, J., K. Xiao, Y. Ke, L. Jiao, C. Hu, Q. Diao, B. Shi, and X. Zou. 2014. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. Poult. Sci. 93:581–588.

Symeon, G. K., F. Mantis, I. Bizelis, A. Kominakis, and E. Rogdakis. 2010. Effects of caponization on growth performance, carcass composition, and meat quality of medium growth broilers. Poult. Sci. 89:1481–1489.

Wales, A. D., V. M. Allen, and R. H. Davies. 2010. Chemical treatment of animal feed and water for the control of *Salmonella*. Foodbor. Pathog. Dis 7:3–15.

Wang, Y., X. Yin, D. Yin, Z. Lei, T. Mahmood, and J. Yuan. 2019. Antioxidant response and bioavailability of methionine hydroxy analog relative to *DL*-methionine in broiler chickens. Anim. Nutr. 5:241–247.

Warton, D. I., S. T. Wright, and Y. Wang. 2012. Distance-based multivariate analyses confound location and dispersion effects. Methods Ecol. Evol 3:89–101.

Wu, Y., X. Yin, Y. Wang, T. Mahmood, M. Shahid, D. Yin, and J. Yuan. 2020. Effect of 2-hydroxy-4-(methylthio) butanoic acid and acidifier on the performance, chyme pH, and microbiota of broilers. Anim. Sci. J 91:e13409.

Zhang, H., S. Pan, K. Zhang, J. Michiels, Q. Zeng, X. Ding, J. Wang, H. Peng, J. Bai, Y. Xuan, Z. Su, and S. Bai. 2020. Impact of dietary manganese on intestinal barrier and inflammatory response in broilers challenged with *Salmonella Typhimurium*. Microorganisms. 8:757.

Zhu, K. H., X. R. Xu, D. F. Sun, J. L. Tang, and Y. K. Zhang. 2014. Effects of drinking water acidification by organic acidifier on growth performance, digestive enzyme activity and caecal bacteria in growing rabbits. Anim. Feed Sci. Tech. 190:87–94.

Zou, L., D. Wang, J. Liu, Y. Bai, Z. Liang, and T. Zhang. 2015. Effects of DL-2-hydroxy-4-(methylthio) butanoic acid on broilers at different dietary inclusion rates. Br. Poult. Sci. 56:337–344.