

Effect of benzoic acid on production performance, egg quality, intestinal morphology, and cecal microbial community of laying hens

Haojie Gong,^{*,1} Zengqiao Yang,^{*,1} Pietro Celi,[†] Lei Yan,[†] Xuemei Ding,^{*} Shiping Bai,^{*} Qiufeng Zeng,^{*} Shengyu Xu,^{*} Zhuowei Su,^{*} Yong Zhuo,^{*} Keying Zhang,^{*} and Jianping Wang^{*,2}

^{*}Animal Nutrition Institute, Key Laboratory of Animal Disease-Resistance Nutrition, Ministry of Education, Sichuan Agricultural University, Chengdu 611130, China; and [†]DSM Nutritional Products, Animal Nutrition and Health, Columbia, MD, USA

ABSTRACT This study was conducted to determine the effects of supplemental dietary benzoic acid on production performance, egg quality, intestinal morphology, and intestinal microbiota of laying hens. A total of seven hundred twenty 45-wk-old Lohman pink-shell laying hens were randomly allocated to 3 dietary treatments: control (CON), diet supplemented with 1,000 mg/kg benzoic acid (BA1), and 2,000 mg/kg benzoic acid (BA2). Each treatment included 10 replicates of 24 hens; laying hens were monitored for 16 wk. Overall, the results indicate that benzoic acid supplementation had no effect on laying rate, feed intake, feed conversion ratio, and breaking rate; however, a decrease in egg weight ($P < 0.01$) was observed in the BA2 group. Albumen height and Haugh unit (HU) were also linearly increased in the BA1 and BA2 groups (linear effect, $P < 0.05$). An increase in duodenum villus height (V) (quadratic effect, $P = 0.041$) and crypt depth (C) (linear effect, $P = 0.012$) was observed in the BA2 group, whereas an increased jejunum C and

decreased V/C (quadratic effect, $P < 0.05$) in the BA1 group. Moreover, an increase in ileum V and C (quadratic effect, $P < 0.05$) was observed in the BA1 group. Microbial richness and diversity were reduced in the BA2 group ($P < 0.01$). An increase in the abundance of *Clostridia* (class), *Clostridiales* (order), *Ruminococcaceae* (family), and *Lachnospiraceae* (family) was noted in the BA1 group, whereas an enrichment of *Bacteroides caecicola* (species) was observed in the BA2 group. The HU positively correlated with genus *Sphaerochaeta* and *Enorma* ($r = 0.56, 0.56; P < 0.05$) but negatively correlated with *Romboutsia*, *Subdoligranulum*, *Helicobacter*, and *Mucispirillum* ($r = -0.58, -0.49, -0.48; -0.70; P < 0.05$). In conclusion, dietary supplementation with benzoic acid had no effect on production performance, but it significantly improved egg quality. In addition, 1,000 mg/kg benzoic acid positively modulated intestinal health by improving intestinal morphology and enriching microbial composition.

Key words: benzoic acid, laying hen, production performance, egg quality, gut microbiota

2021 Poultry Science 100:196–205

<https://doi.org/10.1016/j.psj.2020.09.065>

INTRODUCTION

The addition of antibiotics in poultry diets for nontherapeutic use has been banned in many countries. It has prompted efforts to develop alternatives to antibiotics that include herb products and their derivatives, probiotics, prebiotics, and organic acids. (Cheng et al.,

2014). Acidifiers have been considered as an ideal dietary approach to improve gastrointestinal functionality. Studies have shown that organic acid have the potential to promote growth and intestinal health in grower-finisher pigs and laying hens (Partanen and Mroz, 2005; Kaya et al., 2014). Organic acids are one of the ideal antibiotic alternatives as they can already start working in feed by decreasing its pH value (Diao et al., 2015). This effect not only results in an increase in nutrient digestibility but also in a inhibition of harmful microorganisms in the diet and in the digestive tract (Qin et al., 2007).

Benzoic acid is an organic acidifier, colorless crystalline solid, and the simplest aromatic carboxylic acid (Sim et al., 1955). Benzoic acid and its salts are common

© 2020 Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received May 25, 2020.

Accepted September 7, 2020.

¹These authors contributed to this research work equally.

²Corresponding author: wangjianping@sicau.edu.cn

food preservatives and can also be used as feed additives for some mammals such as pigs (Mroz et al., 2000). It has been reported that the addition of benzoic acid to the diet could increase the weight gain and feed conversion rate of broilers, but excess benzoic acid can have a negative impact on broilers (Józefiak et al., 2007). Also, it has been observed that the combination of benzoic acid and some feed additives consisting of essential oils can improve the performance of broiler (Weber et al., 2012; Giannenas and Papanephytous, 2014). In addition, the report suggests that benzoic acid is safe for laying hens, turkeys, and chickens at 500 mg/kg feed (Rychen et al., 2018); however, it is not known if higher doses might improve gastrointestinal functionality in laying hens.

However, to the best of our knowledge, there are few studies that investigated the effects of the addition of benzoic acid to the diet of laying hens, especially the effect of high level of benzoic acid on gut microbial community of peak-laying hens. Therefore, the aim of this study was to evaluate the effects of supplemental dietary benzoic acid on production performance, egg quality, intestinal morphology, and cecal microbial community of laying hens during peak-laying period.

MATERIALS AND METHODS

Experimental Birds, Diet, and Management

At 45 wk of age, a total of 720 Lohman pink-shell laying hens were randomly allocated into 3 experimental groups that supplemented with 0 mg/kg (CON), 1,000 mg/kg (BA1), and 2,000 mg/kg (BA2) of benzoic acid in the basal diet, respectively. The basal diets were a corn–soybean–type diet and were prepared according to NRC (1994) and Chinese Chicken Breeding Standard (2,004), as shown in Table 1. Each treatment included 10 replicates of 24 laying hens (6 birds/cage). The total experimental period was 16 wk. Before the start of the experimental period, hens were monitored for 4 wk (baseline period), during which the hens were fed the basal diet; production performances during the baseline period were similar between treatments. All hens were housed in an environmentally controlled room where temperature was maintained at approximately 22°C and artificial light by a daily lighting schedule of 16 h light and 8 h dark. Hens were supplied with water and fed a complete feeding mixture in a mash form *ad libitum*.

Productive Performance, Sample Collection, and Chemical Analysis

Egg number, total egg weight, broken eggs, and unqualified eggs (egg weight <50 g or >75 g, misshaped egg, dirty egg, and sand-shelled egg) of each replicate were recorded daily. Daily egg production rate, average egg weight, broken eggs rate, and feed conversion ratio were calculated. Feed conversion ratio was calculated as the ratio of grams of total feed intake to grams of total

Table 1. Composition and nutrient level of basal diet (as-fed basis).

Ingredients	Contents, %
Corn	59.06
Wheat bran	3.87
Soybean oil	1.50
Soybean meal (43% of CP)	15.24
Corn gluten (60% of CP)	5.00
Corn DDGS	5.00
Calcium carbonate (granular)	6.10
Calcium carbonate (powder)	2.50
Calcium hydrophosphate (powder)	0.94
NaCl	0.25
NaHCO ₃	0.10
L-Lysine hydrochloride	0.16
DL-Methionine	0.01
Choline chloride	0.10
Vitamin premix ¹	0.02
Mineral premix ²	0.15
Total	100
Analyzed nutrient content, %	
ME (MJ/kg) ³	11.30
Crude protein	16.00
Calcium	3.70
Total phosphorus	0.60
Available phosphorus ³	0.36
Lysine	0.65
Methionine + Cysteine	0.57

¹ Provided per kilogram of diets: VA 9,950 IU, VB₁ 37.7 mg, VB₂ 12 mg, D-pantothenate 18.2 mg, VB₆ 7.55 mg, VB₁₂ 0.5 mg, VD₃ 5,000 IU, VE 70 IU, VK₃ 4.47 mg, Biotin 4 mg, VC 195 mg, niacin acid 70.35 mg.

² Provided per kilogram of diets: Cu (as copper sulfate) 9.6 mg, Fe (as ferrous sulfate) 64 mg, Mn (as manganese sulfate) 80 mg, Zn (as zinc sulfate) 57 mg, I (as potassium iodide) 0.60 mg, Se (as sodium selenite) 0.36 mg.

³ Calculated according to NRC (1994).

egg weight. Feed intake was recorded weekly. At the end of the experimental period, 30 birds from each treatment were randomly selected (3 birds per replicate). Blood samples were collected from the wing vein into a sterile syringe and then centrifuged at 3,000 × g for 15 min; serum was harvested and stored at –20°C until analysis. After blood sampling, hens were sacrificed by cervical dislocation, and the middle of duodenum, jejunum, and ileum were collected and placed in 10% neutral formaldehyde. The cecum contents were carefully collected, immediately placed in cryogenic vials, and stored at –80°C until they were processed for microbial DNA analysis.

Crude protein (990.03), crude fat without acid hydrolysis (920.39), total phosphorus (965.17), and calcium (984.01) of feed samples were analyzed for by the procedure of AOAC (1995).

Determination of Egg Quality

At the end of 16 wk, 30 eggs were collected from each treatment (3 eggs per replicate). A total of 90 eggs were used to determine egg quality. Eggshell strength was evaluated using an eggshell force gauge model II (Robotmation Co., Ltd., Tokyo, Japan). Yolk color, albumen height, and Haugh units (HU) were evaluated using an egg multi tester (EMT-7300, Robotmation Co., Ltd.). Vernier calipers were used to determine yolk height and yolk diameter, which were then used to calculate the yolk index (yolk index = yolk height/yolk diameter)

Table 2. Effect of dietary supplementation of benzoic acid on production performance of laying hens.

Item	Laying rate, %	Egg weight, g/bird	Feed intake, g/bird/d	FCR, %	Breaking rate, %
CON	93.37	63.86 ^a	116.75	1.95	0.68
BA1	94.03	64.16 ^a	117.64	1.94	0.71
BA2	93.60	63.32 ^b	114.14	1.92	0.50
SEM	0.72	0.24	1.54	0.02	0.11
<i>P</i> -value	0.648	0.007	0.079	0.434	0.165
Linear	0.431	0.431	0.398	0.871	0.491
Quadratic	0.332	0.109	0.120	0.239	0.339

^{a,b}Means with different superscripts within a column differ significantly ($P < 0.05$).

Abbreviations: BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid; CON, control group.

Eggshell thickness was measured on the large end, equatorial region, and small end, using an eggshell thickness gauge (Robotmation Co., Ltd.). Eggshell lightness (L^*), redness (a^*), and yellowness (b^*) were measured as reported by Odabasi et al. (2007).

Intestinal Morphology Analysis

The intestinal segments were flushed clean with and fixed in 10%NoToX (a nonformalin tissue fixative). Histological slides were prepared from 3 cross-sections (5 μ m thick) of each intestinal sample, which were processed in low-melt paraffin and stained with hematoxylin-eosin. The middle of duodenum, jejunum, and ileum (12 cm) were observed with a digital camera microscope (BA400Digital, McAudi Industrial Group Co., Ltd.). The data included villus height (**V**) and crypt depth (**C**); the villus height to crypt depth ratio (**V/C**) was then calculated.

DNA Extraction and Microbiota Analysis

Microbial DNA was extracted from cecum contents using the QIAamp DNA Stool Mini Kit (QIAGEN, CA, Hamburg, Germany) according to the manufacturer's instructions. Total DNA was eluted in 50 μ L of elution buffer and stored at -80°C until measurement in the PCR by LC-Bio Technology (Hang Zhou, China), and the isolation was confirmed by 1.2% agarose gel electrophoresis. Before sequencing, the above 16S rDNA V3-V4 region of each sample was amplified with a set of primers targeting the 16S rRNA gene region. Sequencing libraries were generated using NEB Next Ultra DNA Library Prep Kit for Illumina (New England Biolabs,

Ipswich, MA) following manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and Agilent Bioanalyzer 2,100 system. The library was constructed using the TruSeq DNA PCR-Free Sample Preparation Kit. The constructed library was quantified by Qubit and QPCR. After the library was qualified, the library was sequenced using HiSeq2500 PE250.

Sequencing and bioinformatics analysis were performed by Novogene Bioinformatics Technology Co. (Tianjin, China). Richness and diversity estimations used the α diversity index, including Shannon, Chao1, ACE, and Simpson. Linear discrimination analysis coupled with effect size analysis used the Kruskal-Wallis rank-sum test with a normalized relative abundance matrix to detect features with significantly different abundances between assigned taxa and performs linear discrimination analysis to estimate the effect size of each feature. Linear discrimination analysis coupled with effect size was performed to analyze the bacterial taxa differentially represented between the 2 treatments at different taxonomy levels.

Statistical Analysis

Data were analyzed by one-way ANOVA analysis of variance using GLM procedure of SAS 9.2 software (SAS Institute Inc., Cary, NC). Combined with the Turkey method, multiple comparisons were performed, with $P < 0.05$ as the statistical significance. Also, orthogonal polynomials were used to assess the effect of the dose of BA, and the results were expressed as the mean and SEM.

Table 3. Effect of dietary supplementation of benzoic acid on egg quality of laying hens.

Item	Eggshell color			Egg shape index	Eggshell strength, kg/cm ³	Eggshell thickness, mm-2	Albumen height, mm	Yolk color	Haugh unit	Yolk index
	L^*	a^*	b^*							
CON	83.23 ^a	5.56	16.30	1.32	4.05	36.73	7.13 ^b	13.30	82.66 ^b	0.52
BA1	82.92 ^a	5.50	16.50	1.33	4.20	37.54	7.59 ^a	13.54	85.75 ^a	0.51
BA2	82.39 ^b	5.74	16.55	1.32	4.21	38.14	7.67 ^a	13.65	86.19 ^a	0.52
SEM	0.32	0.22	0.36	0.01	0.14	0.67	0.20	0.15	1.23	0.01
<i>P</i> -value	0.027	0.510	0.756	0.848	0.485	0.114	0.019	0.085	0.009	0.342
Linear	0.032	0.768	0.590	0.432	0.488	0.198	0.021	0.231	0.034	0.544
Quadratic	0.549	0.872	0.425	0.556	0.550	0.310	0.723	0.223	0.651	0.875

^{a,b}Means with different superscripts within a column differ significantly ($P < 0.05$).

Abbreviations: BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid; CON, control group.

Table 4. Effect of dietary supplementation of benzoic acid on intestinal morphology of laying hens.

Item	Duodenum			Jejunum			Ileum		
	Villus height, μm	Crypt depth, μm	V/C	Villus height, μm	Crypt depth, μm	V/C	Villus height, μm	Crypt depth, μm	V/C
CON	1,202.31 ^b	196.08 ^b	6.17	1,142.71	178.71 ^b	6.57 ^b	756.81 ^b	141.46 ^b	5.44
BA1	1,173.56 ^b	204.90 ^b	5.90	1,125.68	221.53 ^a	5.41 ^c	892.46 ^a	162.83 ^a	5.58
BA2	1,343.21 ^a	233.30 ^a	5.93	1,191.25	164.11 ^b	7.43 ^a	704.44 ^b	141.84 ^b	5.18
SEM	43.01	10.7	0.33	31.04	13.71	0.32	30.07	5.90	0.18
<i>P</i> -value	0.005	0.005	0.702	0.070	0.005	0.006	0.007	0.007	0.092
Linear	0.411	0.012	0.671	0.211	0.211	0.012	0.211	0.145	0.128
Quadratic	0.041	0.234	0.459	0.760	0.029	0.027	0.034	0.011	0.231

^{a,b,c}Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviations: BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid; CON, control group.

RESULTS

Production Performance and Egg Quality

Compared with the control group, no significant differences were observed for laying rate, feed intake, feed conversion ratio, and breaking rate in the BA1 and BA2 groups. However, a decrease in egg weight was observed in the BA2 group ($P < 0.01$; Table 2). Compared with the control group, the BA2 decreased significantly L* of eggshell (linear effect, $P = 0.032$; Table 3). Dietary supplementation with benzoic acid increased albumen height (linear effect, $P = 0.021$) and HU (linear effect, $P = 0.034$). No differences among treatments were observed for egg weight, eggshell strength, eggshell thickness, egg shape index, yolk color, and yolk index ($P > 0.05$).

Intestinal Morphology

In the duodenum, V (quadratic effect, $P = 0.041$) and C (linear effect, $P = 0.012$) in the BA2 group compared with the CON and BA1 groups, but no differences in V/C was noted (Table 4). While dietary supplementation with 1,000 mg/kg benzoic acid increased jejunal C ($P < 0.01$), no differences on jejunal V were observed. In the jejunum, V/C was lower in the BA1 group, whereas it was higher in the BA2 group (quadratic effect, $P < 0.05$). In the ileum, we observed that V and C were higher (quadratic effect, $P < 0.05$) in BA1 than CON and BA2 groups. The V/C had not significant differences between 3 groups.

Cecal Microbial Composition

The rarefaction curve for each sample leveled off as the number of sequences increased in all 3 groups, indicating that the number of sequences generated in this study covered much of the extant bacterial diversity present within the hen’s cecum microbiota (Table 5, Figure 1). A total of 10 phyla and 10 genera were found in all samples. The relative microbiota abundances of cecum at phylum level indicated that *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the most abundant phyla in all dietary treatments (CON 96.16%, BA1 94.81%, and BA2 95.70%). No differences in microbial communities, at phylum level, were observed among the treatments group (Table 6). At genus level, we observed that *Bacteroides* was the dominant genus in all dietary treatments. No differences in the relative abundance of the top 10 genera were observed among the treatment groups (Table 7). The shared operational taxonomic units among the 3 treatment groups are shown in Figure 1. Overall, the data indicate that while benzoic acid induced changes in microbial composition in the cecum, it did not alter the dominant species at phylum level.

Alpha Diversity of Cecum Microbiota

As shown in Table 5, the microbial richness and diversity were reflected by the Chao1, ACE, Shannon index, and Simpson index. Chao1 and ACE are indicative of species richness, whereas the Shannon index and Simpson index represent diverse microbial population. The ACE and Shannon index were lower in the BA2 group

Table 5. Effect of dietary supplementation of benzoic acid on alpha diversity index of cecal microbiota laying hens.

Item	Observed species	Shannon	Simpson	Chao1	ACE
CON	632.5 ^{a,b}	6.89 ^{a,b}	0.98	677.42	676.05 ^{a,b}
BA1	656.67 ^a	7.12 ^a	0.98	708.89	713.09 ^a
BA2	586.33 ^b	6.74 ^b	0.98	644.03	641.50 ^b
SEM	12.22	0.06	<0.01	13.45	13.12
<i>P</i> -value	0.042	0.039	0.213	0.144	0.050
Linear	0.326	0.198	0.438	0.623	0.219
Quadratic	0.382	0.037	0.763	0.771	0.013

^{a,b}Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviations: BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid; CON, control group.

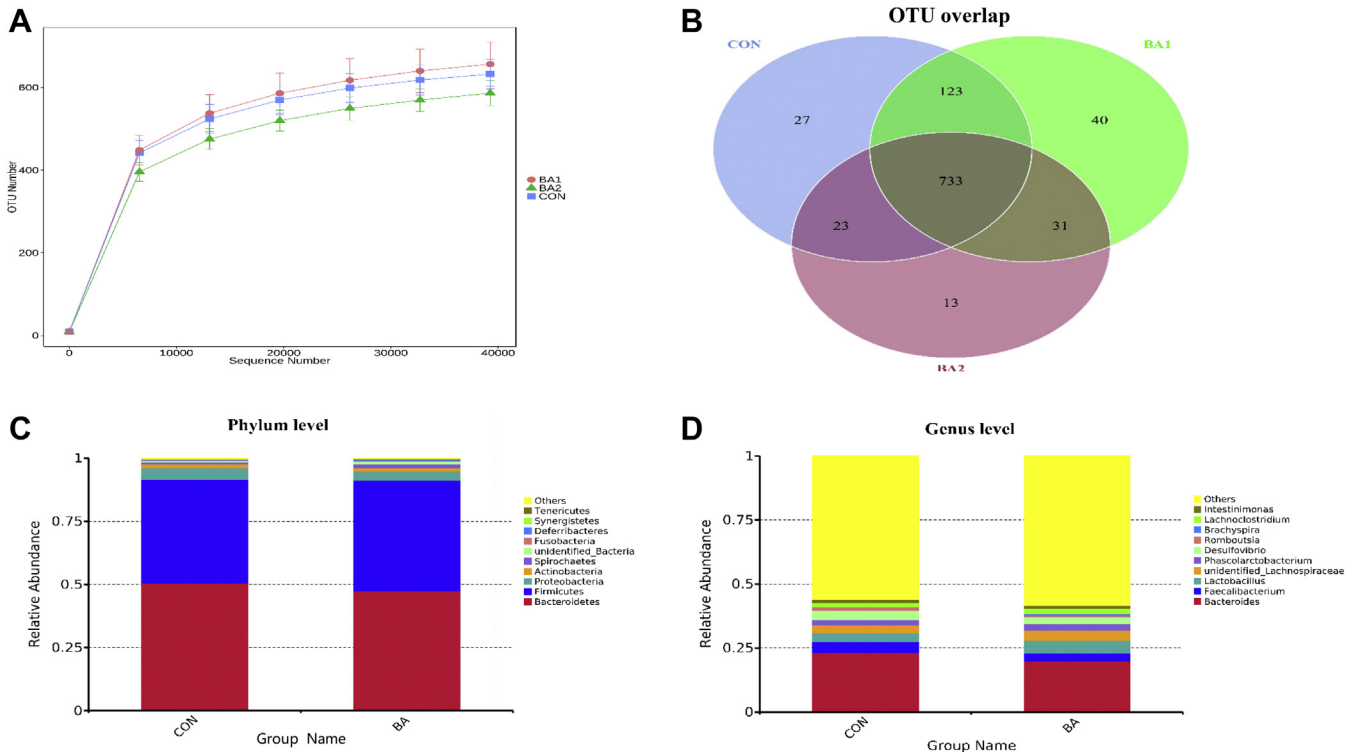


Figure 1. Rank abundance curve of bacterial operational taxonomic units derived from each sample (A). Venn diagram illustrated in cecum microbiota among the samples (B). The relative abundance of the top 10 phylum from samples (C). Bar graph the top 10 genus from samples (D). Abbreviations: CON, control group; BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid.

than those observed in the CON and BA1 ones ($P < 0.05$).

Beta Diversity of Cecum Microbiota

The results indicate that the microbiota of cecal samples was clearly differentiated among the CON and BA2 groups, whereas the separation between the CON and BA1 groups could be hardly detected (Figure 2). The composition of microbiota in the CON group was similar to that observed in the BA1 group than that in the BA2 one. As shown in Figure 3 (linear discrimination analysis coupled with effect size), an increase in the abundance of *Clostridia* (class), *Clostridiales* (order), *Ruminococcaceae* (family), and *Lachnospiraceae* (family) was observed in the BA1 group, whereas an enrichment in *Bacteroides caecicola* (species) was observed in the BA2 group.

Correlations Between Gut Microbiota and Haugh Unit

A Spearman correlation analysis was performed to evaluate the potential link between alterations in gut microbiota composition and HU in laying hens (Figure 4 and Table 8). The HU positively correlated with genus *Sphaerochaeta* and *Enorma* ($r = 0.56, 0.56$; $P < 0.05$), but negatively correlated with *Romboutsia*, *Subdoligranulum*, *Helicobacter*, and *Mucispirillum* ($r = -0.58, -0.49, -0.48, -0.70$; $P < 0.05$).

DISCUSSION

The maintenance of optimal gastrointestinal health and functionality is a key determinant in improving feed efficiency, maintaining animal welfare, and promoting sustainability in animal nutrition (Celi et al., 2017). In recent years, a great deal of research has focused on the development of antibiotic alternatives to maintain or improve poultry health and performance, and several alternatives have been proposed as alternatives to antibiotic growth promoters (Gadde et al., 2017). Benzoic acid is commonly used as feed additive to regulate gastrointestinal functionality (Mao et al., 2019). While its use in broilers has been studied extensively, few reports are available in laying hens.

In this study, dietary supplemented with benzoic acid had no effect on production performance except for a decrease in egg weight observed in group BA2 which received 2,000 mg/kg of benzoic acid. Hassan and Raheem (2016) also observed that feeding benzoic acid to broilers had no significant effect on body weight, weight gain, feed intake, and feed conversion ratio. While it has been reported that organic acid did not influence feed intake in laying hens (Dahiya et al., 2016), it has been shown that organic acid can increase egg weight (Grashorn et al., 2013). The results of this study highlight the inconsistency of the responses to acidifiers which could be because of several factors including the buffering nature of some dietary ingredients and the heterogeneity of the gastrointestinal microbiota (Pearlin et al., 2020). Benzoic acid as an acidifier can promote

Table 6. Effect of dietary supplementation of benzoic acid on cecal microbial relative abundance of the top 10 phylum.

Item	Bacteroidetes	Firmicutes	Proteobacteria	Actinobacteria	Spirochaetes	Unidentified_Bacteria	Fusobacteria	Deferribacteres	Synergistetes	Tenericutes	Others	Firmicutes/Bacteroidetes
CON	50.64	41.06	4.45	1.68	0.57	0.65	0.13	0.29	0.23	0.15	0.14	0.90
BA1	47.55	43.70	3.57	1.48	1.52	1.00	0.32	0.29	0.23	0.17	0.18	0.98
BA2	55.27	37.31	3.12	1.34	1.21	0.83	0.39	0.15	0.15	0.15	0.07	0.69
SEM	2.25	2.09	0.33	0.20	0.23	0.16	0.08	0.05	0.02	0.02	0.02	0.08
<i>P</i> -value	0.394	0.482	0.252	0.810	0.239	0.687	0.459	0.434	0.252	0.933	0.175	0.348
Linear	0.291	0.761	0.188	0.593	0.329	0.544	0.884	0.218	0.211	0.490	0.212	0.551
Quadratic	0.447	0.450	0.297	0.323	0.118	0.211	0.376	0.399	0.557	0.719	0.326	0.770

Abbreviations: BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid; CON, control group.

Table 7. Effect of dietary supplementation of benzoic acid on cecal microbial relative abundance of the top 10 genus.

Item	Bacteroides	Lactobacillus	Faecalibacterium	unidentified_Lachnospiraceae	Phascolarctobacterium	Desulfovibrio	Romboutsia	Brachyspira	Lachnoclostridium	Intestinimonas	Others
CON	23.24	3.44	4.28	3.13	1.99	3.79	1.11	0.03	1.76	1.13	56.1
BA1	19.93	4.87	3.1	4.02	2.55	2.73	0.5	0.64	2.21	1.07	58.47
BA2	25.08	7.26	1.94	2.88	2.58	2.24	0.49	0.37	1.64	0.82	54.68
SEM	1.48	1.01	0.7	0.27	0.26	0.31	0.19	0.19	0.12	0.11	1.24
<i>P</i> -value	0.378	0.315	0.420	0.213	0.611	0.105	0.338	0.469	0.248	0.485	0.477
Linear	0.311	0.651	0.139	0.211	0.442	0.439	0.319	0.590	0.648	0.870	0.713
Quadratic	0.701	0.549	0.178	0.762	0.178	0.177	0.126	0.224	0.218	0.389	0.288

Abbreviations: BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid; CON, control group.

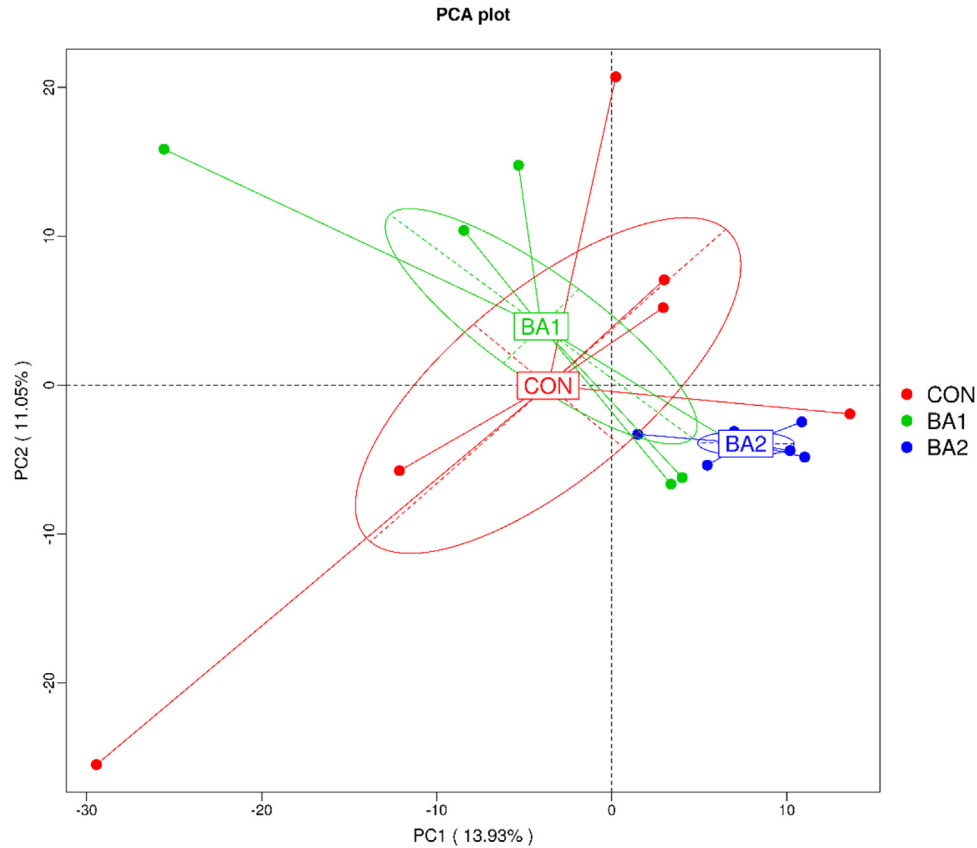


Figure 2. Principal coordinate analysis (PCA) plot of the cecum microbiota based on the unweighted UniFrac metric. Abbreviations: CON, control group; BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid.

the production and activation of digestive enzymes, and it can activate the digestive enzymes via decreasing the pH value in the gastrointestinal tract (Diao et al., 2014; Chen et al., 2015; Mao et al., 2019). While it has been suggested that the optimal level of benzoic acid is 500 mg/kg for laying hens, higher levels did not negatively impact production performance of laying hens (Rychen et al., 2018). Besides, it could be argued that these discrepancies may be related to the form of benzoic acid and associated with the physiological stage of birds, environmental hygiene conditions, and presence of other antimicrobials in the diet (Pearlin et al., 2020).

Eggshell pigmentation is used as a potential indicator for stress and disease conditions in commercial laying hens (Jones et al., 2010). The HU of egg is also an indicator of egg freshness and protein quality (Silversides et al., 1993). In this study, dietary supplemented with benzoic acid increased albumen height and HU and decreased L* of eggshell. It is reported that organic acid had no effect on egg quality parameters such as eggshell strength, eggshell thickness, shape index, yolk color, and HU (Kaya et al., 2015). Yalcin et al. (2009) reported that supplementing the diets of laying hens with 1.0% lactic acid produced significant differences in the albumen index, yolk index, and HU. There are several factors that can affect egg quality, including hen age, strains, nutritional stresses, and housing system. The mechanism of benzoic and organic acid affecting the egg quality of

laying hens is still unclear, and considering the inconsistency in the results reported in the literature, further studies are required to improve our understanding of how organic acids influence egg quality.

Improved gastrointestinal functionality often results in improved digestion and absorption; indeed, the enhancement of nutrient digestibility is associated with the improvement of the bird's the capacity of absorption mainly depends on the absorbing regions of intestinal mucosa. It is important therefore to monitor gastrointestinal functionality to accurately evaluate the impact of nutritional interventions on important aspects such as intestinal mucosa structure and intestinal microbiota composition (Celi et al., 2019). The enhancement of the gut mucosa surface area is beneficial to nutrient transfer from the gut lumen to the bird's circulatory system (Desesso and Jacobson, 2001). The gut surface area is mainly associated with gut mucosal structure, such as intestinal villus morphology, which determines the nutrient absorption capacity of the intestine. The main part of the intestine where nutrients absorption takes place is the small intestinal villus. The longer the V, the larger the absorption area of the small intestine, and the stronger the absorption capacity of nutrients. An increase in C indicates that the villi in the small intestinal mucosa are atrophied and their absorptive capacity is decreased (Zhang et al., 2005). It has been reported that benzoic acid increased V in the duodenum, jejunum

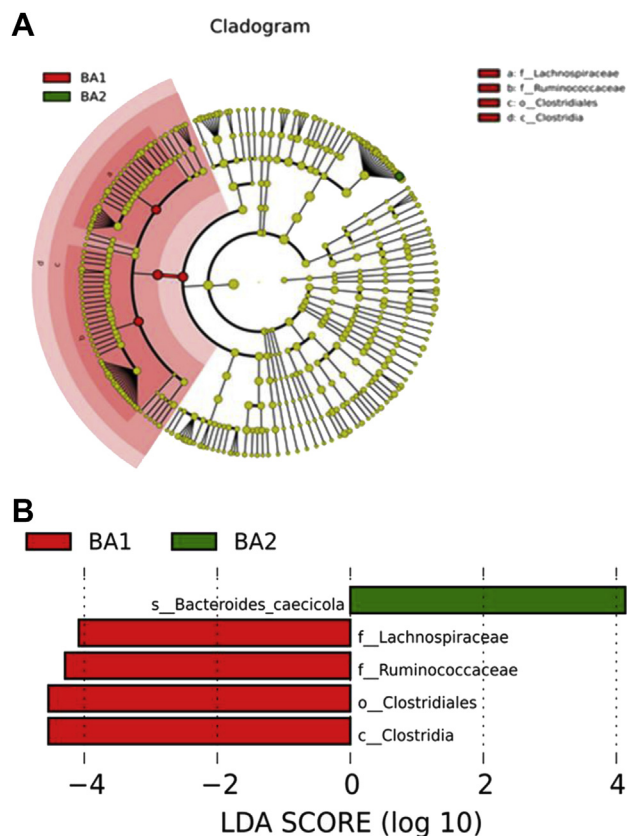


Figure 3. Linear discrimination analysis coupled with effect size (LEfSe) identified the most differentially abundant taxa in the cecum microbiota of different egg laying rate breeders. (A) Taxonomic cladogram obtained from LEfSe analysis of 16SrRNA sequencing. Biomarker taxa are heightened by colored circles and shaded areas. Each circle's diameter is relative to abundance of taxa in the community. (B) Only taxa meeting a linear discrimination analysis significant threshold > 4 are shown. (Red) AP enriched taxa; (Green) LP enriched taxa; (Blue) LPE enriched taxa. Abbreviations: CON, control group; BA1, 1,000 mg/kg benzoic acid; BA2, 2,000 mg/kg benzoic acid.

and ileum in combination with essential oil (Giannenas and Papanephytous, 2014). Similarly, Samanta et al. (2010) reported that in the duodenum, V increased linearly with the dose of dietary organic acids. Positive effects of benzoic acid supplementation on gut mucosal architecture have also been observed in weaned piglets where an increase in ileal V (Halas et al., 2011) and an increase in V and a decrease in C in duodenum, jejunum, and ileum (Diao et al., 2014) has been reported. In the current study, we observed that BA2 increased duodenal V and C and that BA1 increased jejunal V/C and ileal V and C. Our results seem to indicate that the changes in gut mucosal architecture induced by benzoic acid were not consistent across the different segments of the intestinal tract. Moreover, it seems that our observations are also not consistent with the result of previous studies. This apparent discrepancy may be because of the different form and dose of benzoic acid used in other studies to the composition of the organic acid blends used in other studies and to the different animal species. High levels of benzoic acid have been reported to induce

acute or chronic toxicity symptoms, which can seriously impair the health and growth of humans and animals (Amaechi and Anueyiagu, 2012). Overall, in this study, dietary benzoic acid resulted in minor modification of intestinal morphology, and it can be argued that consequently, the absorption capacity of intestinal tract was not affected. This observation might explain the lack of differences in production performances in laying hens supplemented with 1,000 or 2,000 mg/kg of benzoic acid.

The gut microbiota plays a critical role in maintaining normal gastrointestinal and immune function and normal digestion of nutrients (Neu et al., 2007). The diversity of the intestinal microbiota is one of the key determinants of colonization resistance against invading pathogens and high diversity correlates to protection from foreign microorganisms (Kuhn et al., 1993). In this study, we found that dietary supplementation with 1,000 mg/kg of benzoic acid significantly increased cecal microbial richness and diversity. Our observation is in agreement with that of Torrallardona et al. (2006), who found that supplementing benzoic acid in the diet of weaned piglets increased the diversity of the intestinal microbiota. In this study, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the most abundant phyla observed in all experimental groups. Although the microbial communities were not significantly different at phylum level, we noted that *Firmicutes* and *Proteobacteria* decreased when the birds were supplemented with 2,000 mg/kg of benzoic acid. The *Firmicutes/Bacteroidetes* ratio is considered a biomarker of gastrointestinal functionality and can be indicative of eubiosis conditions in the gastrointestinal tract (Cheng et al., 2017). We observed a numerical increase in the *Firmicutes/Bacteroidetes* ratio in the layers that were supplemented with 1,000 mg/kg of benzoic acid, whereas a decrease was noted in the layers that received 2,000 mg/kg of benzoic acid in their diet. It has been suggested that appropriate amounts of benzoic acid are beneficial to intestinal health and may improve the intestinal microbiota (Mao et al., 2019). In this study, we observed that at the genus level, benzoic acid increased the amount of *Lactobacillus*. In agreement with our findings, it has been reported that benzoic acid administration can increase *Lactobacillus* and *Bifidobacterium* population and decrease the numbers of potential harmful microorganisms live coliforms in the cecum of piglets (Kluge et al., 2006). In addition, we found that the genus *Sphaerochaeta* and *Enorma* was positively correlated with HU. The *Sphaerochaeta* is an anaerobic, psychrophilic bacterium (Miyazaki et al., 2014). And the *Sphaerochaeta* genomes are highly enriched in fermentation and carbohydrate metabolism genes (Caro-Quintero et al., 2012). *Enorma* is also an anaerobic bacterium, which interferes with the metabolism of triglycerides, glucose, and glycogen in humans and animals (Mishra et al., 2013). Therefore, it may indicate that the alternation in microbiota such as *Sphaerochaeta* and *Enorma* may affect the metabolism of laying hens.

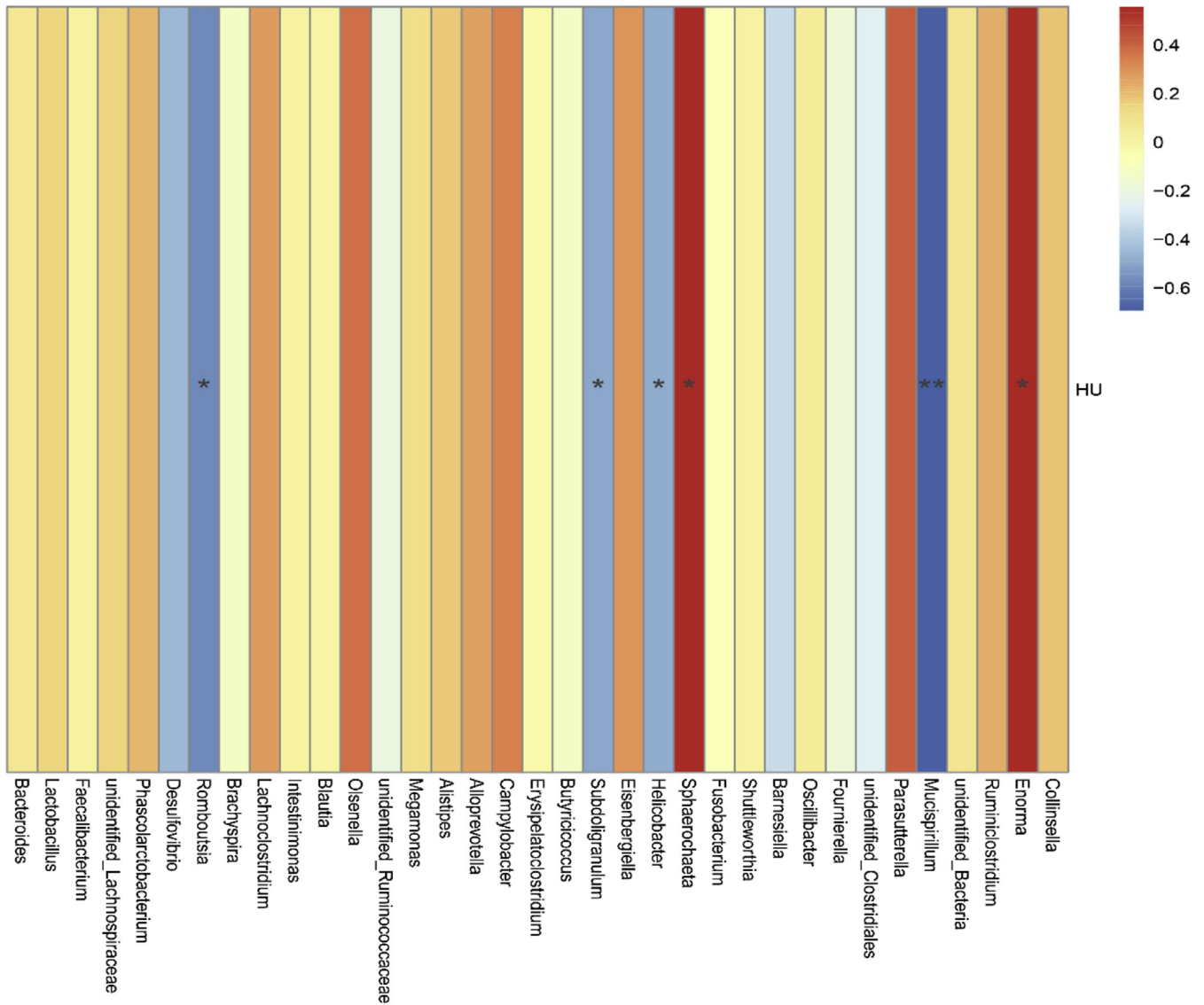


Figure 4. Heatmap of the spearman r correlations between the gut microbiota significantly modified by different Haugh unit at genus level (Top 35). Red indicates positive correlation and blue indicates negative correlation while the color is darker, the correlation is higher. * $P < 0.05$ and ** $P < 0.01$ (following Spearman correlation analysis). Abbreviation: HU, Haugh unit.

Overall, dietary benzoic acid has the potential to promote eubiosis in the cecum of layer hens, and this effect seems to be associated to the level of benzoic acid in the diet.

In conclusion, the moderate amount of benzoic acid had no effect on production performance, but it significantly improved egg quality. The more important point is that benzoic acid improved intestinal morphology and enriched microbial compositions, promoted optimal gastrointestinal health in laying hens. It is not necessary to use high doses of benzoic acid as they negatively influence gastrointestinal health of laying hens.

ACKNOWLEDGMENTS

This work was supported by the earmarked grant for the National Key Research and Development Program of China (Grant No. 2017YFD0500503), National Natural Science Foundation of China (Grant NO. 31872792, 31402031), and Sichuan Provincial Science and Technology Projects (Grant No. 2019YFH0062, 2018NZ20009) for financial support.

DISCLOSURES

The authors declare no conflicts of interest.

Table 8. The spearman r correlations between the gut microbiota significantly modified by different Haugh unit at genus level.

Item	Romboutsia	Subdoligranulum	Helicobacter	Mucispirillum	Sphaerochaeta	Enorma
r-value	-0.58	-0.49	-0.48	-0.70	0.56	0.56
P -value	0.012	0.037	0.042	0.001	0.016	0.017

REFERENCES

- Amaechi, N., and C. F. Anueyiagu. 2012. The effect of dietary benzoic acid supplementation on growth performance and intestinal wall morphology of broilers. *OJAFR* 2:401–404.
- AOAC. 1995. Official Method of Analysis. 16th ed. Assoc. Off. Anal. Chem., Washington, DC.
- Caro-Quintero, A., K. M. Ritalahti, K. D. Cusick, F. E. Loffler, and K. T. Konstantinidis. 2012. The Chimeric Genome of Sphaerochaeta: Nonspiral spirochetes that break with the prevalent dogma in spirochete biology. *MBIO* 3:e00025–12.
- Celi, P., A. J. Cowieson, F. Fru-Nji, R. E. Steinert, A. M. Klünter, and V. Verlhac. 2017. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. *Anim. Feed. Sci. Technol.* 234:88–100.
- Celi, P., V. Verlhac, E. PérezCalvo, J. Schmeisser, and A. M. Klünter. 2019. Biomarkers of gastrointestinal functionality in animal nutrition and health. *Anim. Feed. Sci. Technol.* 250:9–31.
- Chen, J. L., D. W. Chen, B. Yu, H. E. Jun, X. B. Mao, J. Yu, J. Q. Luo, C. Zhang, P. U. Bei, Q. Y. Wang, and P. Zhang. 2015. Effects of benzoic acid on growth performance, organ indexes and gastrointestinal content pH of weaned piglets. *Chin. J. Anim. Nutr.* 1:238–246.
- Cheng, G. Y., H. H. Hao, S. Y. Xie, X. Wang, M. H. Dai, L. L. Huang, and Z. H. Yuan. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Front. Microbiol.* 5:217.
- Cheng, M., X. Zhang, Y. J. Miao, J. X. Cao, Z. F. Wu, and P. F. Weng. 2017. The modulatory effect of (-)-epigallocatechin 3-O-(3-O-methyl) gallate (EGCG3^{Me}) on intestinal microbiota of high fat diet-induced obesity mice model. *Food Res. Int.* 92:9–16.
- Dahiya, R., R. S. Berwal, S. Sihag, C. S. Patil, and Lalit. 2016. The effect of dietary supplementation of salts of organic acid on production performance of laying hens. *Vet. World* 9:1478–1484.
- Desesso, J. M., and C. F. Jacobson. 2001. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food Chem. Toxicol.* 39:209–228.
- Diao, H., P. Zheng, B. Yu, J. He, X. B. Mao, J. Yu, and D. W. Chen. 2014. Effects of dietary supplementation with benzoic acid on intestinal morphological structure and microflora in weaned piglets. *Livest. Sci.* 67:249–256.
- Diao, H., P. Zheng, B. Yu, J. He, X. B. Mao, J. Yu, and D. W. Chen. 2015. Effects of benzoic acid and Thymol on growth performance and gut characteristics of weaned piglets. *Asian Austral. J. Anim.* 28:827–839.
- Gasde, U., W. H. Kim, S. T. Oh, and H. S. Lillehoj. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. *Anim. Health Res. Rev.* 18:26–45.
- Giannenas, I. A., and C. P. Papanophytou. 2014. The effects of benzoic acid and essential oil compounds in combination with protease on the performance of chickens. *J. Anim. Feed. Sci.* 23:73–81.
- Grashorn, M., R. Gruzauskas, A. Dauksiene, V. Jarule, A. Mieželiene, G. Alencikiene, and V. Slausgalvis. 2013. Influence of dietary organic acids on quality and sensory attributes of chicken eggs. *Arch. Geflügelkd.* 77:29–34.
- Halas, D., C. F. Hansen, D. J. Hampson, B. P. Mullan, J. C. Kim, R. H. Wilson, and J. R. Pluske. 2011. Dietary supplementation with benzoic acid improves apparent ileal digestibility of total nitrogen and increases villous height and caecal microbial diversity in weaner pigs. *Anim. Feed. Sci. Tech.* 160:137–147.
- Hassan, R. I. M., and G. S. E. A. Raheem. 2016. Effect of feeding benzoic acid on performance of broiler chickens. *J. Vet. Res.* 4:118–122.
- Jones, D. R., M. T. Musgrove, K. E. Anderson, and H. S. Thesmar. 2010. Physical quality and composition of retail shell eggs. *Poult. Sci.* 89:582–587.
- Józefiak, D., S. Kaczmarek, M. Bochenek, and A. Rutkowski. 2007. A note on effect of benzoic acid supplementation on the performance and microbiota population of broiler chickens. *J. Anim. Feed. Sci.* 16:252–256.
- Kaya, A., H. Kaya, M. Gül, B. A. Yildirim, and S. Timurkaan. 2015. Effect of different levels of organic acids in the diets of hens on laying performance, egg quality criteria, blood parameters, and intestinal histomorphology. *Indian J. Anim. Res.* 49:919–932.
- Kaya, H., A. Kaya, M. Gul, S. Celebi, S. Timurkaan, and B. Apaydin. 2014. Effects of supplementation of different levels of organic acids mixture to the diet on performance, egg quality parameters, serum traits and histological criteria of laying hens. *Eur. Poult. Sci.* 78:12.
- Kluge, H., J. Broz, and K. Eder. 2006. Effect of benzoic acid on growth performance, nutrient digestibility, nitrogen balance, gastrointestinal microflora and parameters of microbial metabolism in piglets. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 90:316–324.
- Kuhn, I., M. Katouli, A. Lund, P. Wallgren, and R. Mollby. 1993. Phenotypic diversity and stability of the intestinal coliform flora in piglets during the first 3 months of age. *Microb. Ecol. Health Dis.* 6:101–107.
- Mao, X. B., Q. Yang, D. W. Chen, B. Yu, and J. He. 2019. Benzoic acid used as food and feed additives can regulate gut functions. *Biomed. Res. Int.* 2019:5721585.
- Mishra, A. K., P. Hugon, J. C. Lagier, T. T. Nguyen, C. Couderc, D. Raoult, and P. E. Fournier. 2013. Non contiguous-finished genome sequence and description of *enorma massiliensis* gen. nov., sp. nov., a new member of the family coriobacteriaceae. *Stand. Genomic Sci.* 8:290–305.
- Miyazaki, M., S. Sakai, K. M. Ritalahti, Y. Saito, Y. Yamanaka, Y. Saito, A. Tame, K. Uematsu, F. E. Loffler, K. Takai, and H. Imachi. 2014. Sphaerochaeta multififormis sp. nov.; an anaerobic, psychrophilic bacterium isolated from seafloor sediment, and emended description of the genus Sphaerochaeta. *Int. J. Syst. Evol. Microb.* 64:4147–4154.
- Mroz, Z., A. W. Jongbloed, K. H. Partanen, K. Vreman, P. A. Kemme, and J. Kogut. 2000. The effects of calcium benzoate in diets with or without organic acids on dietary buffering capacity, apparent digestibility, retention of nutrients, and manure characteristics in swine. *J. Anim. Sci.* 78:2622–2632.
- Neu, J., M. Douglas-Escobar, and M. Lopez. 2007. Microbes and the developing gastrointestinal tract. *Nutr. Clin. Pract.* 22:174–182.
- Odabasi, A. Z., R. D. Miles, M. O. Balaban, and K. M. Portier. 2007. Changes in brown eggshell color as the hen ages. *Poult. Sci.* 86:356–363.
- Partanen, K. H., and Z. Mroz. 2005. Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12:117–145.
- 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.
- Qin, S. T., H. Zhang, X. F. Tang, and Y. Wang. 2007. The Physiology function of acidifier and how to Select acid from different acids. *Chin. J. Anim. Nutr.* 19:515–520.
- Rychen, G., G. Aquilina, G. Azimonti, V. Bampidis, M. D. L. Bastos, G. Bories, A. Chesson, P. S. Cocconcelli, G. Flachowsky, and J. Gropp. 2018. Safety and efficacy of benzoic acid for pigs and poultry. *Efsa J.* 16:5210.
- Samanta, S., T. K. Ghosh, and S. Haldar. 2010. Comparative efficacy of an organic acid blend and bacitracin methylene disalicylate as growth promoters in broiler chickens: effects on performance, gut histology, and small intestinal milieu. *Vet. Med. Int.* 2010:645150.
- Silversides, F. G., F. Twizeyimana, and P. Villeneuve. 1993. Research note: a study relating to the validity of the haugh unit correction for egg weight in fresh eggs. *Poult. Sci.* 72:760–764.
- Sim, G. A., J. M. Robertson, and T. H. Goodwin. 1955. The crystal and molecular structure of benzoic acid. *Acta Crystallogr.* 8:157–164.
- Torrallardona, D., I. Badiola, and J. Broz. 2006. Effects of benzoic acid on performance and ecology of gastrointestinal microbiota in weanling piglets. *Livest. Sci.* 108:210–213.
- Weber, G. M., M. Michalczyk, G. Huyghebaert, H. Juin, C. Kwakernaak, and M. I. Gracia. 2012. Effects of a blend of essential oil compounds and benzoic acid on performance of broiler chickens as revealed by a meta-analysis of 4 growth trials in various locations. *Poult. Sci.* 91:2820–2828.
- Yalcin, S. K., M. T. Bozdemir, and Z. Y. Ozbas. 2009. A comparative study on citric acid production kinetics of two *Yarrowia lipolytica* strains in two different media. *Indian J. Biotechnol.* 8:408–417.
- Zhang, A. W., B. D. Lee, S. K. Lee, K. W. Lee, G. H. An, K. B. Song, and C. H. Lee. 2005. Effects of yeast (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal mucosa development of broiler chicks. *Poult. Sci.* 84:1015–1021.