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Effects of bacitracin zinc, potassium diformate and lauric acid on duodenal digestive functions, intestinal morphology and caecal microflora of broilers

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Abstract: This study investigated the effects of bacitracin zinc, potassium diformate and lauric acid on duodenal digestive functions, intestinal morphology and caecal microflora of broilers. A total of 240 male broilers were randomly allotted to four treatments consisting of five replicates with 12 birds per replicate. The dietary treatments were CON group (basal diet), ANT group (basal diet + 40 mg/kg bacitracin zinc), KDF group (basal diet + 1 000 mg/kg potassium diformate), and LA group (basal diet + 500 mg/kg lauric acid). The results showed that the chymotrypsin activity was significantly enhanced on days 21 and 42 in KDF group compared with CON group ($P < 0.05$), but there were no effects on the activities of trypsin and amylase ($P > 0.05$). No differences in digestive enzyme activities were found between ANT and LA groups ($P > 0.05$). Compared with CON group, the villus length and the villus-to-crypt ratio were significantly increased on days 21 and 42, and the crypt depth was significantly decreased in ANT, KDF and LA groups ($P < 0.05$). Compared with CON group, the supplementation of LA increased the relative abundance of *Faecalibacterium* on day 21 and the relative abundance of *Bacillus* on day 42 in caecum, while the supplementation of KDF increased the relative abundance of *Faecalibacterium* and decreased the relative abundance of *Dorea* on day 42 in caecum ($P < 0.05$). In conclusion, the supplementation of KDF enhanced the chymotrypsin activity. Dietary KDF and LA maintained the intestinal morphology by improving the villus length and the villus-to-crypt ratio and decreasing the crypt depth, and regulated the caecal microflora.

Keywords: broiler; enzyme activity; organic acids; intestinal health

The usage of antibiotics as growth promoters in animal feed was banned in the European Union in 2006, because many negative problems such as drug residues, drug-resistant bacteria and environmental pollution were caused by the irregular and excessive use of antibiotics (Ragaa and Korany

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2016; Wang et al. 2019). Thus, the selection of alternatives to antibiotics has basically become an irresistible trend, especially in the context of the ban on antibiotics. There are many antibiotic substitutes, such as prebiotics, probiotics, essential oils and organic acids, among which organic acids have shown a promising prospect in feeding practices due to the advantages of antibacterial property, enhancing the production performance of animals, and improving the intestinal flora (Emami et al. 2017; Roth et al. 2017). Formic acid has an antimicrobial effect, but the use of formic acid is limited by its pungent odour, corrosiveness and the small molecular weight which can pass directly through the small intestinal epithelium by diffusion, reducing its actions on the posterior intestine (Ragaa and Korany 2016). Potassium diformate (KDF), as a formate, can effectively avoid the disadvantages of formic acid and has the advantages of convenient transportation, low causticity and small irritation, which has attracted interests (Huyghebaert et al. 2011). Meanwhile, the first green feed additive approved by the European Union in 2001 to replace antibiotics to promote growth [Council Regulation (EC) No. 1334/2001] was KDF, mainly due to its strong antimicrobial effect against several bacteria (Zhou et al. 2009).

The medium-chain fatty acid lauric acid (LA) with 12 carbon atoms is mainly found in coconut oil (Kappally et al. 2015). The antimicrobial activity of LA and the derivative monolaurin, which can destroy cell membranes of gram-positive bacteria and lipid-coated viruses, are recognised to interfere with main cellular responses like the activation of transduction cascades and gene transcription (Dayrit 2014). Moreover, most LA is absorbed by epithelial cells in the small intestine and then promotes intestinal integrity (Montgomery et al. 2013).

A large number of reports showed that organic acids played important roles in the intestinal health of poultry (Emami et al. 2017; Roth et al. 2017). However, little is known about the specific study of KDF and LA on the intestinal morphology, digestive enzyme activities and intestinal microflora in broilers. We hypothesised that KDF and LA could improve the intestinal digestive ability, intestinal development and regulate the caecal microflora structure of broilers. Thus, this experiment was conducted to evaluate the effects of KDF and LA on duodenal digestive enzyme activities, intestinal morphology, and caecal microflora of broilers, in order to provide a reference for better utilization of KDF and LA instead of antibiotics in animal production.

MATERIAL AND METHODS

Experimental design, birds and housing

The study was approved by the Committee of Animal Experiments of Fujian Agriculture and Forestry University (approval ID 201912197). A total of 240 one-day-old male Arbor Acre broilers were obtained from Longyan Baotai Agriculture and Animal Husbandry Co., Ltd, China. Birds (41.88 ± 2.57 g of body weight) were allotted to four treatments consisting of five replicates with 12 birds per replicate. The dietary treatments were as follows: CON group (basal diet), ANT group (basal diet + 40 mg/kg bacitracin zinc), KDF group (basal diet + 1 000 mg/kg potassium diformate), LA group (basal diet + 500 mg/kg lauric acid). The supplementation of KDF (95%) and LA (99%) was provided by Shanghai Alliance Biological Technology Co., Ltd, China. The experiment lasted for 42 days and the diet was formulated during starter (days 1–21) and grower (days 22–42) phases, according to the Chinese feeding standard of chickens (2004). The composition and nutrient levels of the experimental diets are shown in Table 1. The poultry house was sterilised and left vacant before the experiment. The room light was provided 24 h/day and the temperature was controlled at 34 °C during the first week, and then gradually reduced by 2 °C every week, and finally kept at about 24 °C. Natural ventilation was used in the house while it was cleaned regularly to keep it clean. Every replicate was reared in one cage (120 cm length × 90 cm width × 40 cm height) with free access to fresh feed in a crumbled form and water. Birds were vaccinated with the combined Newcastle disease virus and infectious bronchitis virus vaccine via intranasal and intraocular administration on days 5 and 10. H5 and H9 inactivated avian influenza vaccines were given to broilers via neck subcutaneous injection on days 5 and 20.

Sample collection

On days 21 and 42 of the trial, the broilers were individually weighed and two broilers close to the

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Table 1. Composition and nutrient levels of basal diets (air-dry basis; %)

Items	Starter (days 1–21)	Grower (days 22–42)
Ingredients		
Corn	51.75	53.25
Soybean meal (CP 43%)	34.73	31.37
Expanded soybean (CP 36.5%)	3.00	6.20
Fishmeal	2.00	0
Soybean oil	4.20	5.00
Limestone	1.22	1.15
$CaHPO_4$	1.54	1.56
DL-Methionine	0.26	0.16
Premix[1]	1.00	1.00
NaCl	0.30	0.30
Total	100.00	100.00
Nutrient levels[2]		
ME (MJ/kg)	12.56	12.98
CP	21.50	20.00
Ca	1.00	0.90
Non-phytate P	0.45	0.40
Lysine	1.19	1.08
Methionine + cysteine	0.91	0.76

CP = crude protein

[1]Premix for days 1–21 (per kg of diet): vitamin A, 9 800 IU; vitamin D_3, 2 780 IU; vitamin E, 19.6 mg; vitamin K_3, 2.24 mg; vitamin B_1, 1.4 mg; vitamin B_2, 7 mg; vitamin B_3, 33.6 mg; vitamin B_5, 9.8 mg; vitamin B_{12}, 0.016 8 mg; pyridoxin, 3.36 mg; biotin, 0.056 mg; folic acid, 1.12 mg; choline, 1 300 mg; Cu, 8 mg; Fe, 100 mg; Mn, 120 mg; Zn, 100 mg; Se, 0.3 mg; I, 0.7 mg. Premix for days 22–42 (per kg of diet): vitamin A, 7 000 IU; vitamin D_3, 2 700 IU; vitamin E, 14 mg; vitamin K_3, 1.6 mg; vitamin B_1, 1 mg; vitamin B_2, 5 mg; vitamin B_3, 24 mg; vitamin B_5, 7 mg; vitamin B_{12}, 0.0168 mg; pyridoxin, 2.40 mg; biotin, 0.04 mg; folic acid, 0.8 mg; choline, 1 000 mg; Cu, 8 mg; Fe, 80 mg; Mn, 100 mg; Zn, 80 mg; Se, 0.3 mg; I, 0.7 mg; [2]calculated values

average replicate weight were chosen and slaughtered. Feed was withdrawn 12 h before slaughter. The middle portion of the duodenum (approximately 4 cm in length) and caecal contents were collected and immediately stored in liquid nitrogen for further analysis. Another duodenum portion was selected, washed with physiological saline and then fixed in the pre-prepared 4% paraformaldehyde solution for 24 h to make a tissue section.

Determination of digestive enzyme activities

The contents of the duodenum were weighed and phosphate buffered saline (pH 7.4) was added by weight/volume = 1 : 5. Then a homogeniser was used to homogenise it fully, the homogenate was centrifuged at 3 000 r/min for 20 min, and the supernatant was taken for the determination of amylase, trypsin and chymotrypsin. The enzymatic activities of amylase, trypsin and chymotrypsin were determined by the double antibody sandwich method using the ELISA kit (Shanghai Enzymatic Biotechnology Co., Ltd, P.R. China). The standard substances with known concen tration and the samples with unknown concentration were added to the microporous enzyme plate, combined with specific antibody followed by a horseradish peroxidase-conjugated antibody specific for the target enzyme to become an antibody-antigen-enzyme antibody complex, then washed and added a tetramethylbenzidine substrate solution. Finally, the microporous enzyme plate was measured for the absorbance at 450 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Take the standard concentration as the horizontal, the optical density values for the vertical, draw the standard curve. The sample concentrations were calculated by the standard curve. All experimental operations were conducted in accordance with the kit instructions.

Observation of intestinal morphology

The process of making duodenal slices was based on the previous operations (Shao et al. 2013). Briefly, the fixed tissue samples were dehydrated by anhydrous ethanol multi-dilution gradient and embedded in paraffin. Paraffin sections (5 μm) were sliced using a rotary microtome, dewaxed, stained with haematoxylin and eosin (HE), and the sheet was sealed. The ten complete and vertically oriented villus heights and the crypt depths from each sample were measured by a microscope, and the ratio of the villus height to the crypt depth was calculated. The villus height was determined from the tip to the base. The crypt depth was determined from the base of the villus to the base of the crypt.

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Determination of caecal microflora

The caecal microflora was analysed by Mata 16S (Wuhan Servicebio Biological Technology Co., Ltd, P.R. China). Microbial genomic DNA was extracted from caecal contents and sequenced using HiSeq2500 PE250 platform (Illumina, San Diego, CA, USA). According to barcode sequence and primer sequence of PCR amplification, all sample data were extracted. Barcode sequence and primer sequence were truncated, and the sample reads were spliced to obtain spliced sequence called original raw tags. Original raw tags were filtered to remove the low-quality, non-length tags and chimeras to get the clean tags of high-quality. Afterwards, the effective tags were analysed by operational taxonomic unit (OTU) using Uparse software (Uparse v7.0.1001; https://drive5.com/uparse/). The species annotation was made on representative sequences of each OTU to obtain corresponding species annotation information and species abundance distribution based on species according to the results of OTU clustering.

Statistical analysis

The data were analysed by one-way ANOVA using SPSS v22.0 (SPSS Inc., Chicago, IL, USA). The model included diet as the fixed effect and block as the random effect. Duncan's test was used for multiple comparisons. Results were expressed as treatment means with their standard error of the mean (SEM). All graphs were generated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of ANT, KDF and LA on duodenal digestive functions of broilers

The effects of dietary supplementation of KDF and LA on duodenal digestive functions of broilers are summarised in Table 2. Compared with CON group, the supplementation of KDF significantly enhanced the chymotrypsin activity on days 21 and 42 ($P < 0.05$), but there were no effects on the activities of trypsin and amylase ($P > 0.05$). The addition of ANT and LA had no effects on digestive enzyme activities ($P > 0.05$). Meanwhile, in broilers fed KDF the chymotrypsin activity was also significantly enhanced compared with those fed ANT on day 42 ($P < 0.05$).

Effects of ANT, KDF and LA on intestinal morphology of broilers

Table 3 presents the effects of KDF and LA on intestinal morphology of broilers. Compared with CON group, the supplementation of ANT, KDF and LA significantly increased the villus length and the villus-to-crypt ratio, while it reduced the crypt depth on days 21 and 42 ($P < 0.05$). Besides, in the villus length there were no differences between ANT, KDF and LA groups on days 21 and 42 ($P > 0.05$). The villus-to-crypt ratio in ANT and KDF groups was higher than that in LA group on days 21 and 42 ($P < 0.05$). Broilers receiving LA showed the higher crypt depth compared with ANT group on day 21 ($P < 0.05$). However, no differences were observed

Table 2. Effects of potassium diformate and lauric acid on duodenal digestive functions of broilers

Items	Treatment diets			
	CON	ANT	KDF	LA
Day 21				
Trypsin (IU/l)	16.94 ± 0.39	17.73 ± 0.94	17.47 ± 0.22	16.70 ± 0.79
Chymotrypsin (IU/l)	27.68 ± 0.56^{b}	30.39 ± 1.50^{ab}	34.39 ± 2.25^{a}	30.18 ± 0.75^{ab}
Amylase (IU/l)	65.19 ± 3.84	69.69 ± 4.60	65.86 ± 4.97	65.64 ± 7.12
Day 42				
Trypsin (IU/l)	19.60 ± 1.39	19.45 ± 0.23	19.58 ± 0.43	19.14 ± 0.34
Chymotrypsin (IU/l)	32.26 ± 1.20^{b}	33.16 ± 1.25^{b}	36.95 ± 0.11^{a}	33.86 ± 0.88^{ab}
Amylase (IU/l)	73.29 ± 3.55	73.85 ± 9.10	74.07 ± 4.36	69.12 ± 4.01

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

^{a,b}The same row with different superscripts means significant difference ($P < 0.05$)

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Table 3. Effects of potassium diformate and lauric acid on intestinal morphology of broilers

Items	Treatment diets			
	CON	ANT	KDF	LA
Day 21				
Villus length (μm)	1 009.00 ± 33.38^{b}	1 199.63 ± 9.10^{a}	1 213.35 ± 6.09^{a}	1 162.58 ± 3.00^{a}
Depth of crypt (μm)	106.97 ± 1.44^{a}	87.02 ± 3.06^{c}	89.12 ± 3.56^{bc}	97.10 ± 1.53^{b}
Villus-to-crypt ratio	9.44 ± 0.38^{c}	13.82 ± 0.57^{a}	13.67 ± 0.63^{a}	11.98 ± 0.18^{b}
Day 42				
Villus length (μm)	1 171.12 ± 5.12^{b}	1 309.11 ± 30.75^{a}	1 359.20 ± 26.84^{a}	1 273.78 ± 45.09^{a}
Depth of crypt (μm)	121.24 ± 3.66^{a}	104.29 ± 1.24^{c}	112.00 ± 2.35^{b}	114.27 ± 0.91^{b}
Villus-to-crypt ratio	9.68 ± 0.30^{c}	12.55 ± 0.25^{a}	12.14 ± 0.30^{a}	11.14 ± 0.31^{b}

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

$^{a–c}$The same row with different superscripts means significant difference ($P < 0.05$)

in the crypt depth in KDF group compared with ANT and LA groups ($P > 0.05$). Broilers fed KDF and LA showed the higher crypt depth compared with ANT group ($P < 0.05$), but there were no differences in the crypt depth between KDF and LA groups on day 42 ($P > 0.05$).

Effects of ANT, KDF and LA on caecal microflora of broilers

The effects of KDF and LA on the alpha diversity of caecal contents microflora are shown in Table 4. Coverage rate almost reached 0.99 among all treatments, which indicated a high coverage. However, there were no differences in the alpha diversity of caecal contents microflora between all treatments on days 21 and 42 ($P > 0.05$).

The relative abundance of caecal microflora at the phylum level in broilers on days 21 and 42 is depicted in Table 5 and Figure 1. Firmicutes were the most abundant phylum on day 21, while Firmicutes and Bacteroidetes were the most abundant phyla on day 42, accounting for 99% and 98% of all bacteria, respectively. Furthermore, the relative abundance of caecal microflora at the phylum level in broilers on days 21 and 42 was not affected by dietary treatments ($P > 0.05$).

Table 4. Effects of potassium diformate and lauric acid on alpha diversity of cecal contents microflora

Items	Treatment diets			
	CON	ANT	KDF	LA
Day 21				
OTU number	351.20 ± 23.09	364.60 ± 23.86	362.00 ± 44.48	384.20 ± 41.01
Ace index	620.86 ± 50.22	640.27 ± 50.53	601.46 ± 83.50	630.81 ± 36.57
Chaol index	619.98 ± 64.47	658.70 ± 71.11	641.52 ± 110.39	650.88 ± 47.18
Shannon index	5.09 ± 0.12	5.20 ± 0.13	4.96 ± 0.71	4.88 ± 0.29
Coverage rate	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 00	0.99 ± 0.00
Day 42				
OTU number	552.60 ± 6.77	638.00 ± 40.69	581.20 ± 49.36	635.40 ± 26.56
Ace index	895.96 ± 13.64	1 124.81 ± 155.66	1 077.77 ± 77.73	1 108.25 ± 91.80
Chaol index	928.08 ± 30.57	1 152.06 ± 142.21	1 103.52 ± 97.80	1 128.58 ± 99.83
Shannon index	4.21 ± 0.15	4.74 ± 0.19	3.21 ± 0.44	4.23 ± 0.24
Coverage rate	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 00	0.99 ± 0.00

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

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Table 5. Effects of potassium diformate and lauric acid on relative abundance of cecal contents microflora at phylum level

Items	Treatment diets			
	CON	ANT	KDF	LA
Day 21				
Actinobacteria	0.02 ± 0.00[a]	0.00 ± 0.00[b]	0.01 ± 0.00[b]	0.01 ± 0.00[b]
Bacteroidetes	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Firmicutes	99.74 ± 0.14	99.52 ± 0.19	99.78 ± 0.80	99.40 ± 0.33
Proteobacteria	0.22 ± 0.15	0.47 ± 0.19	0.20 ± 0.08	0.58 ± 0.34
Other	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
Day 42				
Bacteroidetes	10.48 ± 5.56	14.85 ± 2.07	9.13 ± 3.58	12.70 ± 3.82
Firmicutes	88.69 ± 5.44	84.39 ± 2.30	90.61 ± 3.60	86.58 ± 3.88
Proteobacteria	0.06 ± 0.01	0.13 ± 0.05	0.03 ± 0.02	0.15 ± 0.06
Other	0.77 ± 0.21[a]	0.63 ± 0.14[ab]	0.23 ± 0.06[b]	0.56 ± 0.07[ab]

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

[a,b]The same row with different superscripts means significant difference ($P < 0.05$)

As shown in Table 6 and Figure 2, the dominant bacteria at the genus level in caecum were *Faecalibacterium*, *Oscillospira* and *Ruminococcus* on day 21 while *Faecalibacterium* and *Bacteroidetes* on day 42. Compared with CON group, dietary supplementation of LA increased the relative abundance of *Faecalibacterium* on day 21 and the relative abundance of *Bacillus* on day 42 ($P < 0.05$). In addition, compared with CON group, dietary KDF increased the relative abundance of *Faecalibacterium* and decreased the relative abundance of *Dorea* on day 42 ($P < 0.05$).

DISCUSSION

Effects of ANT, KDF and LA on duodenal digestive functions of broilers

Intestinal digestive enzymes, such as chymotrypsin, trypsin and amylase, are mainly responsible for protein and carbohydrate digestion, and they have great impacts on the growth of broilers (Zhao et al. 2007). Limited reports have shown that the digestive enzyme activities may be closely associated with pH (Kim et al. 2005), but little is known

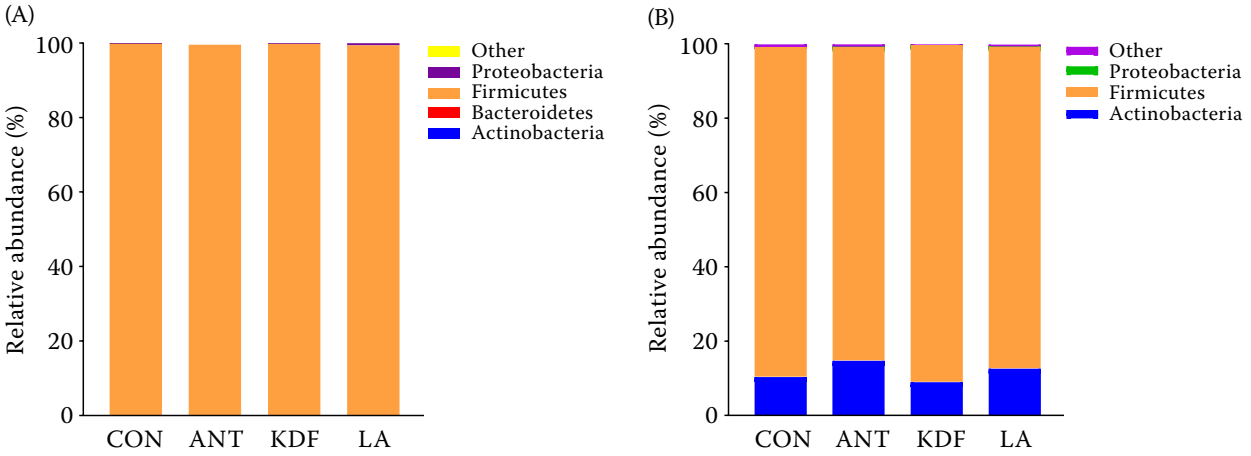

Figure 1. Relative abundance of cecal contents microflora at phylum level (A) on day 21 and (B) on day 42

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

Each bar represents the relative abundance of each treatment. Each color represents a particular bacterial phylum

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Table 6. Effects of potassium diformate and lauric acid on relative abundance of cecal contents microflora at genus level

Items	Treatment diets			
	CON	ANT	KDF	LA
Day 21				
Blautia	0.79 ± 0.31	2.25 ± 0.93	0.41 ± 0.17	0.85 ± 0.33
cc_115	1.24 ± 1.05	1.15 ± 1.14	1.60 ± 0.97	0.27 ± 0.11
Coprobacillus	0.23 ± 0.06	2.06 ± 1.16	0.43 ± 0.17	0.38 ± 0.08
Coprococcus	1.40 ± 0.29	0.71 ± 0.22	2.29 ± 1.43	0.74 ± 0.33
Dorea	1.90 ± 1.00	1.89 ± 0.55	2.20 ± 0.86	2.13 ± 0.65
Faecalibacterium	9.78 ± 3.71^{b}	16.68 ± 2.70^{b}	21.09 ± 5.68^{b}	33.51 ± 2.47^{a}
Lactobacillus	0.24 ± 0.14	0.38 ± 0.44	0.47 ± 0.31	0.26 ± 0.15
Oscillospira	16.75 ± 2.87	16.76 ± 3.23	14.80 ± 1.59	12.23 ± 1.66
Ruminococcus	5.68 ± 1.65	8.59 ± 0.62	6.71 ± 0.89	6.16 ± 0.72
Streptococcus	0.43 ± 0.22	0.32 ± 0.26	0.05 ± 0.04	0.08 ± 0.34
Other	61.56 ± 6.97^{a}	49.22 ± 11.69^{ab}	49.94 ± 12.04^{ab}	43.39 ± 4.57^{b}
Day 42				
Bacillus	0.25 ± 0.12^{b}	0.36 ± 0.12^{ab}	0.10 ± 0.04^{b}	0.62 ± 0.07^{a}
Bacteroides	10.46 ± 5.56	14.78 ± 2.08	9.04 ± 3.58	12.66 ± 3.81
Blautia	0.22 ± 0.05	0.23 ± 0.05	0.19 ± 0.07	0.11 ± 0.02
cc_115	0.57 ± 0.11	0.64 ± 0.21	0.36 ± 0.15	0.52 ± 0.08
Clostridium	0.14 ± 0.03	0.28 ± 0.11	0.11 ± 0.03	0.10 ± 0.04
Coprobacillus	0.37 ± 0.08	0.43 ± 0.16	0.21 ± 0.08	0.35 ± 0.12
Coprococcus	0.79 ± 0.17	0.83 ± 0.14	0.44 ± 0.26	0.62 ± 0.08
Dorea	0.69 ± 0.13^{ab}	0.82 ± 0.14^{a}	0.26 ± 0.10^{c}	0.41 ± 0.07^{bc}
Eggerthella	0.17 ± 0.03^{ab}	0.23 ± 0.06^{a}	0.06 ± 0.01^{b}	0.19 ± 0.03^{a}
Enterococcus	0.23 ± 0.07	0.37 ± 0.17	0.34 ± 0.31	0.10 ± 0.05
Faecalibacterium	46.35 ± 1.88^{b}	37.79 ± 3.89^{b}	63.58 ± 6.52^{a}	45.91 ± 4.05^{b}
Lactobacillus	2.88 ± 0.93	4.42 ± 1.28	1.06 ± 3.46	4.87 ± 1.15
Oscillospira	3.85 ± 0.60	4.63 ± 0.48	4.43 ± 0.21	3.55 ± 0.36
Ruminococcus	1.86 ± 0.33	2.49 ± 0.30	1.62 ± 0.21	1.90 ± 0.21
Streptococcus	0.75 ± 0.42	1.88 ± 0.92	0.17 ± 0.08	0.82 ± 0.37
Other	30.43 ± 4.01^{a}	29.83 ± 2.24^{a}	18.05 ± 3.60^{b}	27.27 ± 2.66^{ab}

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

$^{a–c}$The same row with different superscripts means significant difference ($P < 0.05$)

about the underlying mechanism regarding the use of organic acids to modulate intestinal digestive enzymes. The relevant study mentioned that using organic acids in the diet may reduce the intestinal pH and promote the secretion of intestinal digestive enzymes (Mroz et al. 2002). For example, Naderi Farsani et al. (2020) pointed that using KDF added to rainbow trout diets could potentially enhance the level of lipase, protease, and amylase activity, which was almost in agreement with the above experimental results. The digestive enzyme activities are the highest at the optimal pH (Hamid et al. 2018). The reason why the chymotrypsin activity in KDF group was enhanced was that KDF may rapidly reduce the pH in the intestine and reach the optimal pH to activate chymotrypsin. Adding KDF and LA had no significant improvement in the activities of amylase and trypsin. We speculated that enzyme activities were affected by many factors such as diet composition and regulation of digestive enzymes in the body (Hamid et al. 2018). Thus, the effects of the precise appropriate addition of KDF and LA

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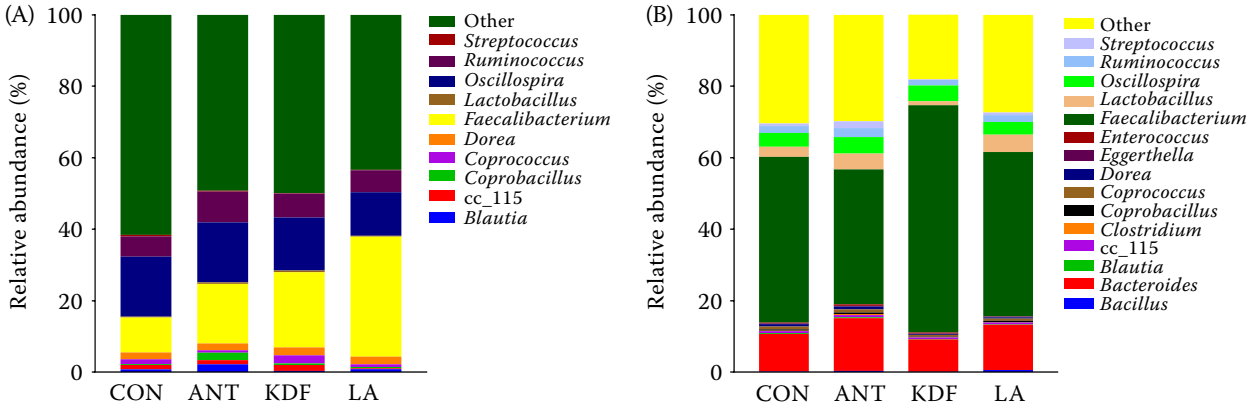

Figure 2. Relative abundance of cecal contents microflora at genus level (A) on day 21 and (B) on day 42

ANT = basal diet + 40 mg/kg bacitracin zinc; CON = basal diet; KDF = basal diet + 1 000 mg/kg potassium diformate; LA = basal diet + 500 mg/kg lauric acid

Each bar represents the relative abundance of each treatment. Each color represents a particular bacterial genus

and underlying mechanisms on intestinal digestive enzymes should be further studied.

Effects of ANT, KDF and LA on intestinal morphology in the duodenum of broilers

Intestinal morphology, including villus height, crypt depth and the villus-to-crypt ratio, is directly connected with the intestinal health of broilers (Sen et al. 2012). In the current experiment, the addition of ANT, KDF and LA could significantly increase the villus length and the villus-to-crypt ratio, decrease the crypt depth in the duodenum, which further promoted the digestion and absorption of nutrients in the intestine. Our results were consistent with earlier findings, where feeding organic acids in broiler diets increased the villus height when compared with the unsupplemented group (Poeikhampha and Bunchasak 2011). Such improved intestinal morphology may be explained by the following reasons. Derivatives of short-chain fatty acids like KDF are the main energy substrate for the growth and development of the intestine (Elala and Ragaa 2015). Most LA is absorbed by the small intestinal epithelial cells, then it is transported into the liver, where it is converted to energy and other metabolites, resulting in promoting the development of small intestinal epithelial cells (Montgomery et al. 2013). Besides, the supplementation of KDF and LA promotes intestinal morphology, which is attributed to their antibacterial effects, reducing the growth and colonization of many pathogenic bacteria, decreasing the inflammation of intestinal mucosa, and increasing the height and secretion of villi (Pelicano et al. 2005). Notably, pH can affect the cell growth and increased acidity is beneficial for the cell division (Lupton and Jacobs 1987). Hence, it can be inferred that KDF and LA could provide energy for intestinal growth, inhibit pathogenic bacteria and reduce pH, and subsequently promote the cell division.

Effects of ANT, KDF and LA on caecal microflora of broilers

Intestinal microflora can not only degrade some substances that cannot be directly used by the body to produce nutrients (Cho et al. 2012), but also maintain the integrity of intestinal structure and act as the first line of defence against pathogenic bacteria invading the body, which play vital roles in animal growth.

The alpha diversity including Chao 1 index, Ace index and Shannon index did not show any differences between the treatments on days 21 and 42, which reflected that KDF and LA exhibited no great influence on the richness of caecal microflora. At the phylum level, Firmicutes were not significantly changed by KDF and LA, but they were still dominant caecal bacteria of broilers, which was consistent with the previous report (Wei et al. 2013). Our results showed that dietary LA increased *Faecalibacterium* on day 21 and *Bacillus* on day 42, while dietary KDF increased the relative abundance of *Faecalibacterium* and decreased the relative abundance of *Dorea* on day 42 compared with CON

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group. Thus, this experiment suggested that dietary KDF and LA regulated the caecal microflora which may be due to the fact that most pathogenic bacteria are suitable for growth in a neutral or weakly alkaline environment, while beneficial bacteria are more suitable for growth in an acidic environment with pH of 5.8~6.2 (Ford 1974). *Faecalibacterium* plays an important role in producing short-chain fatty acids which stimulate epithelial cell proliferation and differentiation and anti-inflammatory effects (Huyghebaert et al. 2011; Huang et al. 2021). Members of the genus *Bacillus* are related to produce antimicrobial compounds such as lipopeptides, surfactin, bacteriocins and bacteriocin-like inhibitory substances (De Cesare et al. 2019). Obesity disease is positively related with *Dorea* (Huang et al. 2021). Therefore, we speculated that the beneficial effects of KDF and LA on the above intestinal morphology might be associated with the regulation of the caecal microflora structure, which was in accordance with the previous report (Gao et al. 2019). Conversely, several studies reported that the addition of KDF and LA reduced intestinal harmful bacteria and beneficial bacteria, or had low effects on intestinal microflora (Canibe et al. 2001; Yang et al. 2019), which was inconsistent with these experimental results, which may be related to the amount of additives, diet composition and different experimental conditions. Actually, most previous findings in broilers fed organic acids were similar to the experimental results. For example, the dietary supplementation of 5 g/kg KDF reduced the number of *Salmonella*, *Escherichia coli* and *Clostridium difficile* in the caecum of Kebao broiler chickens (Ragaa and Korany 2016) whereas there is no clear explanation for the antibacterial mechanisms of organic acids. On the one hand, it is believed that the undissociated form of organic acids is the basic form of their antibacterial action, while the unionised form of organic acids is the fat-soluble form, which can passively diffuse through the microbial cell wall to destroy certain types of bacteria, where they can release protons in a highly alkaline environment, resulting in a decrease in pH in the cell and a decrease in the function of microbial enzymes and nutrient transport system (van der Aar et al. 2017). On the other hand, the cell will consume a large amount of its own energy and transport excessive accumulation of acid ions out of the cell to maintain a balanced intracellular pH, affecting bacterial metabolism (Narayanan et al. 2008). Therefore, organic acids can inhibit harmful bacteria and regulate the balance of intestinal microflora.

CONCLUSION

Based on the current findings, it may be concluded that the addition of KDF enhanced the chymotrypsin activity. Dietary KDF and LA maintained the intestinal morphology by improving the villus length and the villus-to-crypt ratio and decreasing the crypt depth, and regulated the caecal microflora, which are good substitutes for antibiotics.

Conflict of interest

The authors declare no conflict of interest.

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