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Effects of *Clostridium butyricum* and *Enterococcus faecium* on growth performance, lipid metabolism, and cecal microbiota of broiler chickens

Xu Zhao · Yuming Guo · Shuangshuang Guo · Jianzhuang Tan

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Abstract To investigate the effects of *Clostridium butyricum* and *Enterococcus faecium* on the growth performance, lipid metabolism, and cecal microbiota of broilers, 264 one-day-old male Ross 308 broiler chicks were randomly allocated into four treatments with six replicates in a 2×2 factorial arrangement and fed four diets with two levels of *C. butyricum* (0 or 1×10^9 cfu/kg) and two levels of *E. faecium* (0 or 2×10^9 cfu/kg) for 42 days. There was no significant interaction between *C. butyricum* and *E. faecium* on the growth performance, lipid metabolism, and cecal microbiota of broilers. However, broilers supplemented with *E. faecium* had lower (P=0.022) serum leptin level at day 21 and higher (P<0.001) fatty acid synthase (FAS), malic enzyme (ME), and acetyl–CoA carboxylase (ACC) mRNA levels in the liver at day 42. Supplementation of *C. butyricum* improved (P<0.05) the average daily feed intake and average daily gain, increased (P=0.016) the serum insulin level at 21 days of age, enhanced (P<0.05) the content of intramuscular fat, activities of FAS in the liver and lipoprotein lipase (LPL) in the breast muscle, mRNA expression of FAS, ME, and ACC in the liver and LPL in the breast muscle at 42 days of age, but reduced (P=0.030) cecal *Bacteroidetes* relative abundance at 21 days of age. The results of this study indicate that the increased intramuscular fat content of broilers fed *C. butyricum* as observed may be the result of enhanced lipogenesis.

Keywords *C. butyricum* · *E. faecium* · Broiler · Growth · Lipid metabolism · Microbiota

X. Zhao · Y. Guo (✉) · S. Guo · J. Tan
State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, People's Republic of China
e-mail: guoyum@cau.edu.cn

Introduction

The distal gut microorganisms are composed of billions of bacteria and archaea, and *Firmicutes* and *Bacteroidetes* are the two dominant bacterial groups in the broiler cecum (Corrigan et al. 2011; Lu et al. 2003; Zhu et al. 2002). Recent data have revealed that the gut microbiota, especially *Firmicutes* and *Bacteroidetes*, can contribute to host metabolism by several mechanisms including increased energy harvested from the diet, modulation of lipid metabolism, altered endocrine function, and increased inflammatory response (Greiner and Bäckhed 2011). Therefore, supplementation of feed additives to regulate the intestinal microorganisms has become a common practice in the poultry industry. Recently, the use of probiotics has gained increasing interest as a result of the global trend of restricting the use of antibiotics (Lin et al. 2011; Mountzouris et al. 2007; Mountzouris et al. 2010; Samli et al. 2010; Yang et al. 2012).

Clostridium butyricum is a butyric acid-producing, spore-forming, gram-positive anaerobe, which is found in soil and in the intestines of healthy humans and animals (Murayama et al. 1995; Nakanishi and Tanaka 2010). *Enterococcus faecium* is a lactic acid-producing, gram-positive aerobe or facultative anaerobe, which has been used extensively in probiotics (Awad et al. 2009; Böhmer et al. 2005; Patterson and Burkholder 2003). Previous studies demonstrated that both *C. butyricum* and *E. faecium* had positive effects on the growth performance (Samli et al. 2007; Yang et al. 2012), lipid metabolism (Yang et al. 2010; Zhang et al. 2011b), immune function (Yang et al. 2012), gut histomorphology (Samli et al. 2007; Zhang et al. 2011a), and intestinal microbiota (Samli et al. 2007; Samli et al. 2010; Yang et al. 2012; Zhang et al. 2011a) of broilers. However, information is lacking on the effects of *C. butyricum* and *E. faecium* on cecal *Firmicutes* and

Bacteroidetes relative abundance and on the possible interaction between *C. butyricum* and *E. faecium* as feed additives on the growth performance, lipid metabolism, and cecal microbiota of broilers. Therefore, the objectives of this study were to assess the effects of supplementing *C. butyricum* and *E. faecium* on the growth performance, lipid metabolism, and cecal *Firmicutes* and *Bacteroidetes* relative abundance in broiler chickens.

Materials and methods

Experimental design, animals, and management

A total of 264 1-day-old male Ross 308 broiler chicks with similar body weight (BW) (50.22±0.34 g) were obtained from a commercial hatchery (China Agricultural University Breeding Chicken Farm, Hebei, China). Broilers were randomly allocated into 24 wire mesh cages that were then randomly divided into four groups (treatments, six cages per treatment). Each cage had 8,800 (110×80) cm^2 floor space and was equipped with two nipple drinkers and one feeder. Diets contained two supplemental levels of *C. butyricum* (0 or 1×10^9 cfu/kg (500 mg/kg)), and two supplemental levels of *E. faecium* (0 or 2×10^9 cfu/kg (500 mg/kg)) were used in a 2×2 factorial arrangement (denoted as C, CB, EF, and CB+EF, respectively). *C. butyricum* and *E. faecium* were provided by Beijing Gold-tide Biotechnology Co. Ltd., Beijing, China. Previous research conducted in Dr. Y. Guo's laboratory (China Agricultural University, Beijing, China) and in Beijing Gold-tide Biotechnology Co. Ltd. showed that supplementation of *C. butyricum* and *E. faecium* at the levels of 1×10^9 cfu/kg (Yang et al. 2010; Zhang et al. 2011a, b) and 2×10^9 cfu/kg (provided by the manufacturer) in the diet enhanced growth performance or intramuscular fat deposition. Therefore, *C. butyricum* and *E. faecium* were used at 1×10^9 and 2×10^9 cfu/kg, respectively, in the diets in this study. The strains of *C. butyricum* and *E. faecium* used in this study were *C. butyricum* B1 (CGMCC 4845) and *E. faecium* LAB12 (CGMCC 4847), respectively. All experimental diets were in mashed form and were formulated to meet or slightly exceed the nutrient requirements recommended by National Research Council (NRC 1994). Diet compositions are shown in Table 1. All diets were prepared in one batch. Probiotics were first mixed with premix that was subsequently mixed with other ingredients and then stored in covered containers prior to feeding.

Birds were housed in an environmentally controlled room. The temperature was maintained at 35 °C during the first 3 days, between 28 and 30 °C during the subsequent couple of weeks, and at 25 °C during the last 3 weeks of the study. Overhead light was provided continuously for the entire period of the experiment. All birds were vaccinated with the Newcastle disease and infectious bronchitis vaccine (Qian Yuan Hao Biological Co. Ltd., Beijing, China) on days 7 and 21 and with the infectious bursal disease vaccine (Qian Yuan Hao Biological Co. Ltd.) on days 14 and 28. Birds were fed for ad libitum intake and had free access to water throughout the entire experiment.

Body weight and feed intake of broilers of each cage were measured on the morning of days 21 and 42 for determination of average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR). Mortalities and health status were visually observed and recorded daily throughout the entire experimental period. The animal care and use protocol was approved by the China Agricultural University Animal Care and Use Committee (Beijing, China).

Table 1 Ingredients and nutrient composition of experimental diets (% as fed unless noted)

Item	Starter (1 to 21 days)	Grower (22 to 42 days)
Ingredients		
Corn	54.60	60.40
Soybean meal (44.2 % CP)	35.20	30.20
Soy oil	2.65	3.52
Corn gluten meal	3.18	2.00
Calcium hydrogen phosphate	2.00	1.65
Limestone	1.25	1.25
Sodium chloride	0.35	0.35
DL-Met	0.17	0.10
L-Lys HCl	0.08	0.08
Vitamin premix[a]	0.03	0.03
Mineral premix[b]	0.20	0.20
Choline chloride (50 %)	0.26	0.20
Ethoxyquin (33 %)	0.03	0.03
Chemical composition, analyzed		
ME, calculated (kcal/kg)	2,950	3,050
CP	21.50	19.00
Calcium	1.00	0.91
Available phosphorus	0.46	0.40
Lys	1.15	1.01
Met	0.50	0.40
TSAA	0.84	0.71
Thr	0.82	0.73
Trp	0.25	0.23

[a] Supplied per kilogram of diet: vitamin A, 12,500 IU; cholecalciferol, 2,500 IU; vitamin E, 30 IU; vitamin K_3, 2.65 mg; thiamin, 2 mg; riboflavin, 6 mg; pantothenic acid, 12 mg; cobalamin, 0.025 mg; niacin, 50 mg; biotin, 0.0325 mg; and folic acid, 1.25 mg

[b] Supplied per kilogram of diet: Mn, 100 mg; Fe, 80 mg; Zn, 75 mg; Cu, 8 mg; I, 0.35 mg; and Se, 0.15 mg

Sample collection

Blood samples were taken from the wing vein of six birds (one bird per cage) of each treatment on the morning of days 21 and 42 of the experiment (after being fasted for 12 h with free access to water) using sterilized needles and nonheparinized tubes. The blood samples were incubated at 37 °C for 2 h and were then centrifuged at 1,500×*g* for 10 min at 4 °C. The resultant serum (supernatant) was stored in 0.5-mL Eppendorf tubes at −20 °C. After bleeding, the same birds used in the blood sampling were fed for ad libitum intake and had free access to water for approximately 2 h and then slaughtered by exsanguination under deep sodium pentobarbitone anesthesia (30 mg/kg BW, i.v.). Tissue samples were obtained from the liver, breast muscle, and thigh muscle. Some of them were washed with ice-cold isotonic physiological saline (0.9 %, *w*/*v*, NaCl), immediately frozen in liquid nitrogen, and stored at −40 °C for analysis of enzyme activity, and some of the tissues were immediately stored in RNAfixer (BioTeke Co. Ltd, Beijing, China), preserved at 4 °C overnight, and transferred to −80 °C for subsequent extraction of total RNA. Cecal contents placed in sterilized tubes were quickly frozen in liquid nitrogen and stored at −80 °C for DNA extraction. Abdominal fat, breast muscle, and thigh muscle were collected as described by Yang et al. (2010). Abdominal fat was calculated as percentage of abdominal fat weight relative to the total weight of eviscerated and abdominal fat. Intramuscular fat content was determined by extraction with diethyl ether in a Soxhlet apparatus (method 960.39; AOAC 1990) and expressed as a permillage of fresh muscle weight.

Determination of serum hormones levels and biochemical parameters

Insulin (INS), growth hormone (GH), leptin (LEP), free triiodothyronine (FT3), and free thyroxine (FT4) levels in the serum were determined with the commercial RIA kits obtained from Beijing North Institute of Biological Technology (Beijing, China) according to the manufacturer's protocol.

Concentrations of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and non-esterified fatty acid (NEFA) in the serum were measured by colorimetric enzymatic methods using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Enzyme activity assays

Liver samples were homogenized with ice-cold buffer solution (20 mM Tris–HCl, 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol; pH 7.4) in the ratio of 1:2 for 2 min with Ultra-turrax (T10 basic, IKA, Germany). The homogenate was then centrifuged at 100,000×*g* and 4 °C for 1 h. The resultant supernatants were subsequently stored at −40 °C and used to analyze fatty acid synthase (FAS, EC 2.3.1.85) and malic enzyme (ME, EC 1.1.1.40) activities. The activities of FAS and ME were measured according to the method described by Cai et al. (2009). The activities of FAS and ME were expressed as units per milligram of protein for liver. One unit of FAS activity was defined as the amount of enzyme per milligram of tissue protein that would catalyze the conversion of 1 nmol of NADPH to $NADP^+$ in 1 min. One unit of ME activity was defined as the amount of enzyme per milligram of tissue protein that would catalyze the conversion of 1 nmol of $NADP^+$ to NADPH in 1 min.

Breast muscle and thigh muscle samples were assayed for activities of lipoprotein lipase (LPL, EC 3.1.1.34) and hormone-sensitive lipase (HSL, EC 3.1.1.3). LPL was determined using the same procedure as described by Wang et al. (2010) with assay kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China), and HSL was measured using the same method as described by Huang et al. (2006). The activity of LPL was expressed as units per milligram of protein for breast muscle or thigh muscle. One unit of LPL activity was defined as 1 μmol FFA released from 1 mg of muscle tissue protein per hour. The activity of HSL was expressed as units per milligram of protein for breast muscle or thigh muscle. One unit of HSL activity was defined as the amount of enzyme per milligram of tissue protein that would hydrolyze 1 nmol of triglyceride at 37 °C in 1 min.

Real-time quantitative PCR analysis of gene expression

Total RNA of the liver, breast muscle, or thigh muscle was extracted using TRIzol reagen (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was assessed via agarose gel electrophoresis. For the production of cDNA, 1 μg of total RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. All of the cDNA preparations were stored frozen at −30 °C until further use. A real-time quantitative PCR assay was performed with the 7500 Real Time PCR Systems (Applied Biosystems, Foster City, CA, USA) according to optimized PCR protocols using a SYBR-Green PCR kit (Applied Biosystems). The thermocycle protocol consisted of 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 1 min annealing/extension at 60 °C. The gene-specific primers for FAS, ME, ACC, LPL, and GAPDH are listed in Table 2. Standard curves were derived from serial dilutions of samples. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve

Table 2 Gene-specific primer of the lipid metabolism-related enzyme

Gene	Genbank number	Primer position	Primer sequences(5′→3′)	Product size (bp)	Reference
FAS	J03860	Forward Reverse	CCAACGATTACCCGTCTCAA CAGGCTCTGTATGCTGTCCAA	170	Zhang et al. (2011b)
ME	NM204303	Forward Reverse	TGCCAGCATTACGGTTTAGC CCATTCCATAACAGCCAAGGTC	175	Cai et al. (2009)
ACC	NM_205505	Forward Reverse	AATGGCAGCTTTGGAGGTGT TCTGTTTGGGTGGGAGGTG	136	Cai et al. (2009)
LPL	NM_205282	Forward Reverse	CAGTGCAACTTCAACCATACCA AACCAGCCAGTCCACAACAA	150	Cai et al. (2009)
GAPDH	NM_204305	Forward Reverse	GGTGAAAGTCGGAGTCAACGG CGATGAAGGGATCATTGATGGC	108	Druyan et al. (2007)

FAS fatty acid synthase, *ME* malic enzyme, *ACC* acetyl–CoA carboxylase, *LPL* lipoprotein lipase

analysis and subsequent agarose gel electrophoresis. The ΔΔCt method was used to estimate mRNA abundance. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene, and the mRNA expression of target genes was normalized to GAPDH mRNA expression. All of the samples were analyzed in triplicate, and the mean values of these measurements were used for the calculation of mRNA expression.

Real-time PCR quantification of the predominant bacterial divisions

DNA of the cecal contents was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final elution volume was 200 μL, and concentration and purity were determined spectrophotometrically at an optical density of 260 and 280 nm (Eppendorf AG 22331, Hamburg, Germany). The real-time quantitative PCR assay was performed and subjected to the same assay conditions for tissue samples (400 nM of each of the forward and reverse primers and 0.25 ng DNA for each reaction). The amplifying primer sets of *Firmicutes*, *Bacteroidetes*, and total bacteria are listed in Table 3. Abundance of these microbes was expressed as a proportion of total estimated cecum bacterial 16S rDNA according to the equation: relative quantification = $2^{-(\mathrm{Ct\ target}-\mathrm{Ct\ total\ bacteria})}$, where Ct represents threshold cycle (Chen et al. 2008; Mao et al. 2010).

Data calculations and statistical analyses

The experiment was conducted as a completely randomized design with treatments arranged factorially. The statistical model included the main effects of *C. butyricum*, *E. faecium*, and the interactions. Effects of treatments were analyzed as a 2×2 factorial arrangement by two-way ANOVA. Duncan's multiple-range test was used for multiple comparisons when a significant interaction was detected. All statements of significance were based on $P<0.05$, and tendencies were indicated if the P value was between 0.05 and 0.10. Cage was considered as the experimental unit. All statistical analyses were performed using the GLM procedure of Statistical Analysis System (SAS) 8.1 software (SAS Institute, Inc., Cary, NC, USA).

Results

Growth performance and fat deposition

All broilers appeared healthy throughout the entire experimental period (data not shown). Birds in the *C. butyricum*-supplemented groups had higher ($P<0.05$) ADFI and ADG in either phase or the entire period of the experiment and higher ($P<0.05$) breast muscle and thigh muscle intramuscular fat content at 42 days of age (Table 4). However, growth performance and fat

Table 3 Primers used for the quantification of the predominant bacterial divisions' expression by real-time PCR

Target species	Primer name and sequence (5′→3′)	Approximate size (bp) of product	Reference
Firmicutes	Firm934F, GGAGYATGTGGTTTAATTCGAAGCA Firm1060R, AGCTGACGACAACCATGCAC	126	Guo et al. (2008a)
Bacteroidetes	Bact934F, GGARCATGTGGTTTAATTCGATGAT Bact1060R, AGCTGACGACAACCATGCAG	126	Guo et al. (2008a)
Total bacteria	Eub338F, ACTCCTACGGGAGGCAGCAG Eub518R, ATTACCGCGGCTGCTGG	200	Fierer et al. (2005)

Table 4 Effect of *C. butyricum* and *E. faecium* on growth performance and fat deposition

Item	Treatment				SEM	CB, mg/kg		EF, mg/kg		Main effect (*P*-value)		
	C	CB	EF	CB+ EF		0	500	0	500	CB	EF	CB× EF
1 to 21 days[a]												
ADFI (g/day)	40.6	42.1	41.2	42.6	0.68	40.9b	42.3a	41.3	41.9	0.042	0.414	0.966
ADG (g/day)	24.3	25.9	25.0	25.4	0.77	24.7	25.6	25.1	25.2	0.219	0.873	0.425
FCR (g/g)	1.68	1.63	1.65	1.68	0.038	1.66	1.66	1.66	1.66	0.880	0.813	0.338
22 to 42 days[a]												
ADFI (g/day)	125.5	132.2	127.6	135.2	2.31	126.5b	133.7a	128.8	131.4	0.005	0.273	0.833
ADG (g/day)	67.8	71.4	69.6	73.8	1.49	68.7b	72.6a	69.6	71.7	0.016	0.174	0.853
FCR (g/g)	1.85	1.85	1.83	1.83	0.021	1.84	1.84	1.85	1.83	0.968	0.368	0.968
1 to 42 days[a]												
ADFI (g/day)	83.0	87.1	84.4	88.9	1.35	83.7b	88.0a	85.1	86.6	0.005	0.255	0.865
ADG (g/day)	46.0	48.7	47.3	49.6	0.82	46.7b	49.1a	47.3	48.5	0.007	0.189	0.835
FCR (g/g)	1.77	1.74	1.74	1.76	0.017	1.75	1.75	1.75	1.75	0.804	0.804	0.190
21 days[b]												
Abdominal fat (%)	1.16	1.22	1.13	1.16	0.111	1.14	1.19	1.19	1.14	0.667	0.699	0.905
Intramuscular fat (breast muscle) (mg/g)	1.92	1.99	1.86	1.91	0.223	1.89	1.95	1.95	1.88	0.782	0.770	0.973
Intramuscular fat (thigh muscle) (mg/g)	3.85	3.90	3.47	4.10	0.634	3.66	4.00	3.88	3.78	0.597	0.884	0.652
42 days[b]												
Abdominal fat (%)	1.96	2.00	1.92	1.90	0.150	1.94	1.95	1.98	1.91	0.934	0.657	0.856
Intramuscular fat (breast muscle) (mg/g)	4.08	7.21	4.60	7.23	0.714	4.34b	7.22a	5.64	5.92	<0.001	0.708	0.730
Intramuscular fat (thigh muscle) (mg/g)	3.54	6.93	5.23	7.46	1.066	4.38b	7.20a	5.23	6.35	0.016	0.309	0.591

Means within a row followed by different letters differ significantly ($P<0.05$)

C diet without *C. butyricum* and *E. faecium* supplementation, *CB* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) but without *E. faecium* supplementation, *EF* diet with *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) but without *C. butyricum* supplementation, *CB+EF* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) and *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) supplementation, *CB C. butyricum*, *EF E. faecium*, *CB×EF* interaction between *C. butyricum* and *E. faecium* treatment

[a] Data are means for six replicates of 11 chicks per cage

[b] Data are means for six replicates of one chick per cage

deposition of birds in either phase or the entire period of the experiment were not significantly affected by the addition of *E. faecium*. There was also no interaction between *C. butyricum* and *E. faecium* on these parameters.

Serum hormone levels

There were no significant interactions between *C. butyricum* and *E. faecium* for INS, GH, LEP, and FT3 levels in the serum at both 21 and 42 days of age (Table 5). However, supplementation of *C. butyricum* increased (P=0.016) INS level in the serum at 21 days of age and tended (P=0.065) to decrease GH level in the serum at 42 days of age. Addition of *E. faecium* reduced (P=0.022) LEP level and tended (P=0.071) to decrease GH level in the serum at 21 days of age. There was a significant interaction (P=0.008) between *C. butyricum* and *E. faecium* on FT4 level in the serum at 42 days of age. The serum FT4 level in *C. butyricum*- or *E. faecium*-supplemented groups at 42 days of age was lower ($P<0.05$) than that of broilers in the non-probiotic-supplemented group.

Serum biochemical parameters

There were no significant main effects of *C. butyricum* and *E. faecium* and no significant interaction between *C. butyricum* and *E. faecium* on the concentrations of TC, HDL-C, LDL-C, and NEFA in the serum at both 21 and 42 days of age (Table 6). However, supplementation of *C. butyricum* tended (P=0.077) to decrease the concentration of TG in the serum at 21 days of age.

Table 5 Effect of *C. butyricum* and *E. faecium* on serum hormone

Item	Treatment				SEM	CB, mg/kg		EF, mg/kg		Main effect (*P*-value)		
	C	CB	EF	CB+EF		0	500	0	500	CB	EF	CB×EF
21 days[a]												
Insulin (μIU/mL)	3.23	3.77	2.97	4.14	0.326	3.10b	3.96a	3.50	3.56	0.016	0.860	0.350
Growth hormone (ng/mL)	1.07	1.03	0.88	0.84	0.099	0.98	0.94	1.05	0.86	0.677	0.071	1.000
Leptin (ng/mL)	0.54	0.51	0.44	0.40	0.041	0.49	0.45	0.52a	0.42b	0.366	0.022	0.874
Free triiodothyronine (ng/mL)	5.61	4.28	5.69	5.27	0.555	5.65	4.77	4.95	5.48	0.129	0.350	0.422
Free thyroxine (ng/mL)	10.02	11.16	10.69	11.59	0.624	10.35	11.38	10.59	11.14	0.117	0.391	0.846
42 days[a]												
Insulin (μIU/mL)	3.25	3.45	3.15	2.35	0.457	3.20	2.90	3.35	2.75	0.520	0.204	0.283
Growth hormone (ng/mL)	1.12	0.82	1.20	1.06	0.114	1.16	0.94	0.97	1.13	0.065	0.183	0.499
Leptin (ng/mL)	0.36	0.37	0.30	0.30	0.056	0.33	0.33	0.36	0.30	0.941	0.263	0.848
Free triiodothyronine (ng/mL)	2.30	2.26	2.48	2.46	0.275	2.39	2.36	2.28	2.47	0.917	0.506	0.979
Free thyroxine (ng/mL)	10.94a	9.28b	8.50b	9.28b	0.409	9.72	9.28	10.11	8.89	0.292	0.008	0.008

Means within a row followed by different letters differ significantly ($P<0.05$)

C diet without *C. butyricum* and *E. faecium* supplementation, *CB* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) but without *E. faecium* supplementation, *EF* diet with *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) but without *C. butyricum* supplementation, *CB+EF* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) and *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) supplementation, *CB C. butyricum*, *EF E. faecium*, *CB×EF* interaction between *C. butyricum* and *E. faecium* treatment

[a] Data are means for six replicates of one chick per cage

Lipogenic enzyme activities

There were no significant main effects of *C. butyricum* and *E. faecium* at 21 days of age and no significant interaction between *C. butyricum* and *E. faecium* at both 21 and 42 days of age on the activities of FAS and ME in the liver and of LPL and HSL in the breast muscle and thigh muscle (Table 7). However, birds supplemented with *C. butyricum* had higher ($P=0.036$) activity of FAS in the liver and higher ($P=0.011$) activity of LPL in the breast muscle at 42 days of age.

Lipid metabolism gene expression

There were no significant main effects of *C. butyricum* and *E. faecium* at 21 days of age and no significant interaction between *C. butyricum* and *E. faecium* at both 21 and 42 days of age on FAS, ME, and ACC mRNA levels in the liver and LPL mRNA levels in the breast muscle and thigh muscle (Table 8). However, birds supplemented with *C. butyricum* had higher ($P<0.05$) FAS, ME, and ACC mRNA levels in the liver and higher ($P=0.035$) LPL mRNA level in the breast muscle at 42 days of age. Addition of *C. butyricum* tended ($P<0.10$) to increase LPL mRNA levels in the breast muscle at 21 days of age and in the thigh muscle at 42 days of age. Supplementation of *E. faecium* increased ($P<0.001$) FAS, ME, and ACC mRNA levels in the liver at 42 days of age and tended ($P=0.083$) to increase LPL mRNA level in the breast muscle at 21 days of age.

Quantitation of cecal microbiota

There was no significant interaction between *C. butyricum* and *E. faecium* on the relative abundance of *Firmicutes* and *Bacteroidetes* in the cecal contents at both 21 and 42 days of age (Table 9). Birds in the *C. butyricum*-supplemented groups had lower ($P=0.030$) *Bacteroidetes* relative abundance in the cecal contents at 21 days of age. However, the relative abundance of *Firmicutes* and *Bacteroidetes* at both 21 and 42 days was not significantly affected by the addition of *E. faecium*.

Discussion

Effect of *C. butyricum* and *E. faecium* on growth performance and fat deposition in broilers

In recent years, higher growth performance and better body composition with higher intramuscular fat and lower abdominal fat have gained increasing interest. The results of this study showed that ADFI, ADG, and intramuscular fat in the breast and thigh muscle of the 42-day experimental period were increased by *C. butyricum* supplementation at

Table 6 Effect of *C. butyricum* and *E. faecium* on serum biochemical parameters of broilers

Item	Treatment				SEM	CB, mg/kg		EF, mg/kg		Main effect (*P*-value)		
	C	CB	EF	CB+EF		0	500	0	500	CB	EF	CB×EF
21 days[a]												
Total cholesterol (mM)	3.67	3.66	3.78	3.56	0.134	3.73	3.61	3.67	3.67	0.392	0.995	0.447
Triglyceride (mM)	0.60	0.43	0.51	0.48	0.053	0.55	0.45	0.51	0.49	0.077	0.709	0.200
High-density lipoprotein cholesterol (mM)	3.20	3.33	3.32	3.25	0.108	3.26	3.29	3.26	3.29	0.814	0.837	0.370
Low-density lipoprotein cholesterol (mM)	0.75	0.71	0.70	0.70	0.037	0.73	0.71	0.73	0.70	0.595	0.403	0.595
Non-esterified fatty acid (μM)	539.5	473.5	484.4	473.7	58.66	512.0	473.6	506.5	479.1	0.521	0.645	0.643
42 days[a]												
Total cholesterol (mM)	3.47	3.48	3.40	3.47	0.126	3.44	3.47	3.47	3.43	0.794	0.754	0.814
Triglyceride (mM)	0.54	0.58	0.54	0.58	0.047	0.54	0.58	0.56	0.56	0.417	0.958	0.986
High-density lipoprotein cholesterol (mM)	2.99	3.14	2.95	3.03	0.083	2.97	3.09	3.06	2.99	0.177	0.400	0.679
Low-density lipoprotein cholesterol (mM)	0.62	0.59	0.63	0.61	0.055	0.62	0.60	0.61	0.62	0.695	0.833	0.904
Non-esterified fatty acid (μM)	1,034.6	982.8	1,000.0	945.1	112.06	1,017.3	964.0	1,008.7	972.6	0.639	0.750	0.989

Means within a row followed by different letters differ significantly ($P<0.05$)

C diet without *C. butyricum* and *E. faecium* supplementation, *CB* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) but without *E. faecium* supplementation, *EF* diet with *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) but without *C. butyricum* supplementation, *CB+EF* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) and *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) supplementation, *CB C. butyricum*, *EF E. faecium*, *CB×EF* interaction between *C. butyricum* and *E. faecium* treatment

[a] Data are means for six replicates of one chick per cage

a level of 1×10^9 cfu/kg of diet, but FCR and abdominal fat were not affected. These data demonstrated that broilers consuming a diet containing 1×10^9 cfu/kg of *C. butyricum* had improved growth performance and body composition. However, the similar ADFI, ADG, FCR, intramuscular fat, and abdominal fat of broilers fed diets with or without *E. faecium* in this study indicated that supplementation of *E. faecium* at the level of 2×10^9 cfu/kg had no effect on growth performance and fat deposition. Yang et al. (2012) observed that supplementation of *C. butyricum* at dietary levels of 2×10^7 or 3×10^7 cfu/kg significantly improved the growth performance of broilers. However, diets containing 1×10^9 cfu/kg of *C. butyricum* did not affect the abdominal fat but markedly increased the intramuscular fat of broilers at 42 days of age (Yang et al. 2010). In contrast, Zhang et al. (2011a) observed no effect on the growth performance in broilers when *C. butyricum* was fed at a level of 1×10^9 cfu/kg. Samli et al. (2007) observed that diets containing 2×10^{10} cfu/kg of *E. faecium* improved growth performance, whereas supplementation of *E. faecium* at the level of 3.5×10^8 cfu/kg of diet decreased the feed intake (Samli et al. 2010). The discrepancy among these studies may be attributed to several factors such as broiler breeds and physiological stages, diet compositions, administration levels, and the methods of probiotic supplementation. The enhanced growth performance and intramuscular fat of broiler in *C. butyricum*-supplemented groups observed in this study may be partially attributed to a healthy microecological environment which alters metabolism by increasing digestive enzyme activity, producing large amounts of short-chain fatty acids, such as butyric acid and acetic acid, and promoting immune function (Yang et al. 2012; Zhang et al. 2011a).

Effect of *C. butyricum* and *E. faecium* on serum hormone levels and serum biochemical parameters in broilers

Fat deposition in adipose tissue, which represents a balance between fat synthesis and fat degradation, is regulated by a variety of hormones (Chilliard 1993; Huang et al. 2006; Miao et al. 2008). Numerous studies established that INS, GH, LEP, FT3, and FT4 have a marked effect on lipid metabolism (Huang et al. 2006; Ma et al. 2008; Miao et al. 2008). Chung et al. (1983) and Dunshea et al. (1992) reported that the rate of lipogenesis is acutely stimulated by INS, which regulated the rate of glucose conversion to lipid. Hence, the higher INS level in groups supplemented with *C. butyricum* in the current study suggests a greater capacity of broilers to synthesize fat. Growth hormone can contribute to a decrease in lipogenic enzyme activities (e.g.,

Table 7 Effect of *C. butyricum* and *E. faecium* on lipogenic enzymes activities

Item	Treatment				SEM	CB, mg/kg		EF, mg/kg		Main effect (*P*-value)		
	C	CB	EF	CB+EF		0	500	0	500	CB	EF	CB×EF
21 days[a]												
Liver fatty acid synthase (U/mg protein)	1.46	1.62	1.87	1.42	0.187	1.67	1.52	1.54	1.65	0.441	0.574	0.120
Liver malic enzyme (U/mg protein)	1.58	1.40	1.56	1.50	0.334	1.57	1.45	1.49	1.53	0.724	0.910	0.863
Breast muscle lipoprotein lipase (U/mg protein)	0.11	0.14	0.13	0.22	0.036	0.12	0.17	0.12	0.17	0.116	0.159	0.415
Thigh muscle lipoprotein lipase (U/mg protein)	1.02	0.93	1.09	0.91	0.102	1.05	0.92	0.98	1.00	0.205	0.809	0.675
Breast muscle hormone sensitive lipase (U/mg protein)	1.37	1.50	1.43	1.43	0.072	1.40	1.47	1.44	1.43	0.357	0.946	0.368
Thigh muscle hormone sensitive lipase (U/mg protein)	2.35	2.45	2.50	2.52	0.148	2.42	2.49	2.40	2.51	0.673	0.452	0.789
42 days[a]												
Liver fatty acid synthase (U/mg protein)	1.10	1.80	1.15	1.58	0.252	1.13b	1.69a	1.45	1.37	0.036	0.759	0.598
Liver malic enzyme (U/mg protein)	1.05	1.66	1.80	1.87	0.374	1.43	1.76	1.36	1.84	0.381	0.216	0.476
Breast muscle lipoprotein lipase (U/mg protein)	0.11	0.13	0.11	0.12	0.005	0.11b	0.13a	0.12	0.12	0.011	0.729	0.304
Thigh muscle lipoprotein lipase (U/mg protein)	0.20	0.18	0.19	0.18	0.013	0.19	0.18	0.19	0.19	0.309	0.700	0.797
Breast muscle hormone sensitive lipase (U/mg protein)	1.46	1.37	1.42	1.52	0.096	1.44	1.44	1.41	1.47	0.980	0.579	0.339
Thigh muscle hormone sensitive lipase (U/mg protein)	2.10	2.14	2.15	2.04	0.101	2.13	2.09	2.12	2.10	0.727	0.826	0.492

Means within a row followed by different letters differ significantly ($P<0.05$)

C diet without *C. butyricum* and *E. faecium* supplementation, *CB* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) but without *E. faecium* supplementation, *EF* diet with *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) but without *C. butyricum* supplementation, *CB+EF* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) and *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) supplementation, *CB C. butyricum*, *EF E. faecium*, *CB×EF* interaction between *C. butyricum* and *E. faecium* treatment

[a] Data are means for six replicates of one chick per cage

FAS) as well as a decline in insulin sensitivity and insulin-stimulated lipogenesis (Etherton 2000; Louveau and Gondret 2004), thereby leading to a decrease in lipogenesis and consequent reduction in adipose tissue mass. Leptin is a paracrine functioning hormone, produced by adipose tissue (Ramsay et al. 1998), that can affect feed intake and energy metabolism and subsequently act on the tissue to alter lipid accretion by altering lipolysis and lipogenesis (Ramsay 2004; Sun et al. 2006). Thyroid hormones are mainly considered as hormones involved in the catabolic processes of lipids (Sheridan and Kao 1998). Thyroid hormones influence all major metabolic pathways. Their most obvious and well-known action is to increase in basal energy expenditure by acting on protein, carbohydrate, and lipid metabolism. With regard to lipid metabolism, thyroid hormones can affect the synthesis, mobilization, and degradation of lipids, and degradation is influenced more than synthesis (Pucci et al. 2000). Therefore, levels of GH, LEP, FT3, and FT4 in the serum is highly correlated with fat deposition and serve as signals reflecting the body fat content. The lower levels of LEP and FT4 in groups supplemented with *C. butyricum* and *E. faecium* in this study indicated the higher capacity of broilers to synthesize fat. The observed effects of *C. butyricum* supplementation on enhancing intramuscular fat in this study may be partially attributed to the enhanced INS level, reduced FT4 level, and unchanged GH, LEP, and FT3 levels in the serum by *C. butyricum*.

Concentrations of serum lipids and lipoproteins are indicative of the metabolic regulations in a steady state and, especially, of the basal adjustment of fatty acid circulation between the adipose tissue and the liver (Mossab et al. 2002). Non-esterified fatty acid is mainly generated by the hydrolysis of TG in adipose tissue, and its concentration reflects the lipolytic activity in adipose tissue (Mersmann and MacNeil 1985). The similar TC, TG, HDL-C, LDL-C, and NEFA in the serum among all groups of broilers in this study indicated that supplementation of *C. butyricum* and *E. faecium* had no effect on serum lipids and lipoproteins. Zhang et al. (2011b) observed that diet containing 1×10^9 cfu/kg of *C. butyricum* did not affect the concentrations of TG, TC, HDL-C, and LDL-C in the serum of broilers.

Table 8 Effect of *C. butyricum* and *E. faecium* on mRNA abundance of lipogenic genes

Item	Treatment				SEM	CB, mg/kg		EF, mg/kg		Main effect (*P*-value)		
	C	CB	EF	CB+EF		0	500	0	500	CB	EF	CB×EF
21 days[a]												
Liver fatty acid synthase	1.00	1.31	1.76	1.28	0.340	1.30	1.38	1.16	1.52	0.807	0.296	0.259
Liver malic enzyme	1.00	1.09	1.34	0.93	0.185	1.01	1.17	1.05	1.14	0.396	0.629	0.186
Liver acetyl–CoA carboxylase	1.00	1.46	1.42	1.26	0.246	1.21	1.36	1.23	1.34	0.548	0.671	0.219
Breast muscle lipoprotein lipase	1.00	2.09	2.11	2.55	0.333	1.48	2.35	1.55	2.39	0.089	0.083	0.451
Thigh muscle lipoprotein lipase	1.00	1.18	1.10	1.14	0.398	1.05	1.16	1.09	1.12	0.789	0.930	0.865
42 days[a]												
Liver fatty acid synthase	1.00	3.94	8.97	13.28	0.705	4.99b	8.61a	2.47b	11.13a	<0.001	<0.001	0.344
Liver malic enzyme	1.00	3.61	10.21	14.48	1.527	5.61b	9.04a	2.30b	12.35a	0.036	<0.001	0.593
Liver acetyl–CoA carboxylase	1.00	1.65	2.10	2.45	0.211	1.55b	2.05a	1.32b	2.28a	0.029	<0.001	0.484
Breast muscle lipoprotein lipase	1.00	1.69	0.96	1.11	0.175	0.98b	1.37a	1.31	1.04	0.035	0.108	0.159
Thigh muscle lipoprotein lipase	1.00	1.06	0.80	1.79	0.278	0.90	1.39	1.03	1.25	0.079	0.364	0.119

Means within a row followed by different letters differ significantly ($P<0.05$)

C diet without *C. butyricum* and *E. faecium* supplementation, *CB* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) but without *E. faecium* supplementation, *EF* diet with *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) but without *C. butyricum* supplementation, *CB+EF* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) and *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) supplementation, *CB C. butyricum*, *EF E. faecium*, *CB×EF* interaction between *C. butyricum* and *E. faecium* treatment

[a] Data are means for six replicates of one chick per cage

Capcarova et al. (2010) reported that supplementation of *E. faecium* also did not affect the TG and TC in the serum of broilers. It appeared that the serum biochemical parameter response observed in this study was similar to those of broilers in previous studies. These results may be related to the homeostatic mechanism of broilers.

Effect of *C. butyricum* and *E. faecium* on lipogenic enzyme activities and lipid metabolism gene expression

In chickens, de novo lipogenesis occurs primarily in the liver, and most of the body's endogenous lipids are of hepatic origin (Hermier 1997; O'Hea and Leveille 1968). There is considerable evidence that ACC plays a role in the

Table 9 Effect of *C. butyricum* and *E. faecium* on microbial populations (% of total bacterial 16S rDNA)

Item	Treatment				SEM	CB, mg/kg		EF, mg/kg		Main effect (*P*-value)		
	C	CB	EF	CB+EF		0	500	0	500	CB	EF	CB×EF
21 days[a]												
Firmicutes	35.97	38.62	36.66	33.08	1.559	36.32	35.30	37.15	34.71	0.789	0.179	0.090
Bacteroidetes	6.01	2.70	5.08	4.64	0.712	5.55a	3.86b	4.54	4.84	0.030	0.533	0.087
42 days[a]												
Firmicutes	32.19	32.47	30.74	27.49	2.278	31.47	29.98	32.33	29.12	0.520	0.174	0.447
Bacteroidetes	10.46	8.59	9.03	13.43	2.088	9.75	11.02	9.53	11.23	0.550	0.424	0.149

Means within a row followed by different letters differ significantly ($P<0.05$)

C diet without *C. butyricum* and *E. faecium* supplementation, *CB* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) but without *E. faecium* supplementation, *EF* diet with *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) but without *C. butyricum* supplementation, *CB+EF* diet with *C. butyricum* (1×10^9 cfu/kg (500 mg/kg)) and *E. faecium* (2×10^9 cfu/kg (500 mg/kg)) supplementation, *CB C. butyricum*, *EF E. faecium*, *CB×EF* interaction between *C. butyricum* and *E. faecium* treatment

[a] Data are means for six replicates of one chick per cage

regulation of fatty acid biosynthesis in animal tissues and is generally considered as a rate-limiting enzyme of lipogenesis in animals (Numa et al. 1970). Fatty acid synthase, which catalyzes the last step in the lipogenic pathway, is also a key determinant for the maximal capacity of a tissue to synthesize fatty acid. Furthermore, FAS and ACC seem to be coordinately regulated (Toussant et al. 1981). Nicotinamide adenine dinucleotide phosphate (NADPH) is a coenzyme required for the reductive biosynthesis of fatty acid, and ME is recognized to be the main enzyme involved in supplying NADPH (Wise and Ball 1964; Young et al. 1964). Therefore, it is well recognized that FAS, ME, and ACC are three main indicators that reflect de novo lipogenesis (Cai et al. 2009; Huang et al. 2008). The higher activities of FAS and ME and mRNA levels of FAS, ME, and ACC in the liver in groups supplemented with *C. butyricum* in this study indicated the greater capacity of broilers to synthesize fatty acid. However, supplementation of *E. faecium* had no effect on the activities of FAS and ME in the liver, suggesting that the up-regulated FAS and ME transcription may not be consistent with the activities of FAS and ME in the liver.

Lipoprotein lipase, located on the capillary endothelium of extrahepatic tissue, catalyzes the rate-limiting step in the hydrolysis of TG from circulating CM and VLDL in adipose tissues (Goldberg 1996). High expression of muscle LPL has been shown to be associated with increased intramuscular triacylglycerol accumulation and fat deposition (Voshol et al. 2001). Therefore, the observed effects of *C. butyricum* supplementation on increasing intramuscular fat may be partially attributed to the enhanced FAS, ME, and ACC mRNA levels in the liver and the increased LPL mRNA level in the muscle by *C. butyricum*. Supplementation of *E. faecium* did not affect the intramuscular fat of broilers, which is likely due to the unchanged lipogenic enzyme activities in the tissues.

Hormone-sensitive lipase is the key enzyme catalyzing the hydrolysis of stored TG in adipose tissue into FFA and glycerol (Huang et al. 2006). Hence, HSL is a main indicator reflective of lipodieresis in broiler adipose tissue. The similar activity of HSL among all groups in the present study suggests that supplementation of *C. butyricum* and *E. faecium* had no effect on TG degradation. The quantity of triglyceride in adipose tissue is the net outcome of synthesis and degradation (Liu et al. 2007; Sanz et al. 2000). Therefore, the increased intramuscular fat content of broilers fed *C. butyricum* is likely associated with the enhanced lipogenesis and unchanged lipodieresis. However, the mechanisms by which the *C. butyricum* and *E. faecium* increased the activities of lipogenic enzymes or mRNA abundance of lipogenic genes in tissues are not clear.

Information on the effects of *C. butyricum* and *E. faecium* on the activities of lipogenic enzymes and mRNA abundance of lipogenic genes in tissues is lacking. Zhang et al. (2011b) observed that diets containing 1×10^9 cfu/kg of *C. butyricum* increased the activity of LPL in the breast muscle without affecting FAS activity and mRNA level in the liver and LPL mRNA level in the breast muscle. The discrepancy between the two studies is likely due to the different broiler breeds and diet compositions.

Effect of *C. butyricum* and *E. faecium* on quantitation of cecal microbiota of broilers

The cecum is the main site of fermentation in the gastrointestinal tract. It harbors a diverse microbial community. Recently, the use of probiotics as intestinal microbiota regulators has gained increasing interest because of the global trend of restricting the use of antibiotics. Yang et al. (2012) observed that diets containing 2×10^7 or 3×10^7 cfu of *C. butyricum*/kg decreased *Escherichia coli* (14 and 42 days), *Salmonella*, and *Clostridium perfringen* (14 to 42 days) but increased *Lactobacillus* and *Bifidobacterium* (21 to 42 days) in the cecal contents of broilers. Zhang et al. (2011a) also demonstrated that *C. butyricum* supplemented at the level of 1×10^9 cfu/kg enhanced lactic acid bacteria in the cecal contents at 40 days of age. Furthermore, Samli et al. (2010) reported that supplementation of *E. faecium* at a level of 3.5×10^8 cfu/kg positively influenced the ileal and cecal microbiota, with a significant decrease in the population of *E. coli*. In the current study, the reduced *Bacteroidetes* relative abundance in the cecal contents of *C. butyricum*-supplemented broilers at 21 days of age showed that supplementation of *C. butyricum* at the level of 1×10^9 cfu/kg of diet alters the microbiota in the cecum. The increased acetic acid, butyric acid, valeric acid, and total short-chain fatty acids in the cecal contents and the reduced pH of cecal contents by *C. butyricum* supplementation have favorable effects on cecal microorganisms (Yang et al. 2012; Zhang et al. 2011a), which may have partially contributed to the reduced *Bacteroidetes* relative abundance in the cecal contents. However, the similar *Firmicutes* relative abundance in the cecal contents among all groups of broilers in this study may be due to a large number of genera and species with widely different metabolic capacities in the *Firmicutes* division or the number of animals. The gut microbiota of animals mainly consists of the *Firmicutes* and *Bacteroidetes* divisions (Eckburg et al. 2005; Ley et al. 2006), and their relative abundances are closely related to the amount of body fat in humans, mice, and pigs (Guo et al. 2008a, b; Ley et al. 2005, 2006; Turnbaugh et al. 2006). Therefore, the observed effects of *C. butyricum* supplementation on increasing intramuscular fat content, enhancing serum insulin level, and increasing lipogenic enzyme activities and lipid metabolism genes expression in tissues may be partially attributed to the reduced *Bacteroidetes* relative abundance in the cecal contents by *C. butyricum*.

Information on the effects of probiotics on the relative abundance of *Firmicutes* and *Bacteroidetes* in the cecal contents of broilers is lacking. Angelakis and Raoult (2010) observed that inoculation with *Lactobacillus* sp. at a level of 4×10^{10} bacteria/animal increased the number of *Firmicutes* in the feces, while the number of *Bacteroidetes* in the feces remained stable, and only the second *Lactobacillus* sp. inoculation decreased the population of *Bacteroidetes*. The discrepancy between the two studies may be attributed to several factors such as broiler breeds and physiological stages, diet compositions, administration levels, and methods of probiotic supplementation.

In conclusion, supplementation of *C. butyricum* at a level of 1×10^9 cfu/kg in diets enhanced growth performance by increasing ADFI and ADG without affecting FCR throughout the duration of the study, improved fat deposition by increasing breast muscle and thigh muscle intramuscular fat content at 42 days of age, enhancing serum insulin level at 21 days of age and increasing lipogenic enzymes activities and lipid metabolism genes expression in tissues at 42 days of age, and regulated intestinal microbes by reducing *Bacteroidetes* relative abundance without affecting *Firmicutes* relative abundance in the cecal contents at 21 days of age. Supplementation of *E. faecium* at 2×10^9 cfu/kg enhanced lipogenesis by decreasing serum leptin at 21 days of age and increasing FAS, ME, and ACC mRNA levels in the liver at 42 days of age. However, there was no interaction between *C. butyricum* and *E. faecium* on the growth performance, lipid metabolism, and cecal microbiota of broilers.

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