Effects of *Clostridium butyricum* on breast muscle lipid metabolism of broilers

Xu Zhao\(^a\), Xiao Ding\(^b\), Zaibin Yang\(^b\), Yiru Shena, Shan Zhang\(^a\) and Shourong Shi\(^a\)

\(^a\)Poultry Institute, Chinese Academy of Agricultural Sciences, Yangzhou, Jiangsu, China; \(^b\)College of Animal Sciences and Technology, Shandong Agricultural University, Tai-an, Shandong, China

**ABSTRACT**

To investigate the effects of *Clostridium butyricum* (*C. butyricum*) on breast muscle lipid metabolism of broilers, 192 one-day-old male Arbor Aces broilers were randomly allocated into 2 treatments with 6 replicates in a completely randomised design. The broilers were fed corn-soybean meal-based diets and supplemented with 0 or 1 × 10⁹ cfu of *C. butyricum*/kg of diet for 42 days. The birds in the *C. butyricum*-supplemented group showed higher (*p < .05*) average daily gain during the grower phase and throughout the entire period of the experiment and a lower (*p = .047*) feed conversion rate during the grower phase. Supplementation with *C. butyricum* increased (*p < .05*) the intramuscular fat content, lipoprotein lipase activity and mRNA levels in the breast muscle at 42 days of age, increased (*p = .032*) the serum insulin level at 21 days of age, and enhanced (*p = .020*) the caecal *Firmicutes* relative abundance at 42 days of age. Additionally, supplementation with *C. butyricum* reduced (*p < .05*) the serum growth hormone levels at both 21 and 42 days of age, decreased (*p < .05*) the ileum angioptielin-like protein 4 (*ANGPTL4*) mRNA levels and serum *ANGPTL4* concentrations at 42 days of age, and decreased (*p < .05*) the hormone-sensitive lipase activity, carnitine palmitoyltransferase 1, carnitine palmitoyltransferase 2, and long-chain acyl-CoA dehydrogenase mRNA levels in the breast muscle at 42 days of age. In conclusion, dietary supplementation with *C. butyricum* could potentially target caecal microbiota and reduce the breast muscle fatty acid oxidation of broilers.

**Introduction**

Distal gut microorganisms are composed of billions of bacteria and archaea, and *Firmicutes* and *Bacteroidetes* are the two dominant bacterial phyla in the broiler caecum (Zhu et al. 2002; Lu et al. 2003; Corrigan et al. 2011). In the past decade, there is a growing speculation that intestinal microbiota can influence fat storage (Greiner and Bäckhed 2011). One circulating factor that communicate between the intestinal microbiota and other parts of the body is angiopoietin-like protein 4 (*ANGPTL4*), which is a circulating lipoprotein lipase (*LPL*) inhibitor and plays a key role in regulating fatty acid oxidation in the skeletal muscle of the host (Greiner and Bäckhed 2011). Therefore, supplementation with feed additives to regulate the intestinal microbiota may be a good way to achieve the goal of high skeletal muscle intramuscular fat in broilers without affecting abdominal fat in the poultry industry. Additionally, the use of probiotics has gained increasing interest recently as a result of the global trend to restrict the use of antibiotics (Mountzouris et al. 2007, 2010; Samli et al. 2010; Lin et al. 2011; Yang et al. 2012).

*Clostridium butyricum* (*C. butyricum*) is a butyric-acid-producing, spore-forming, Gram-positive anaerobe that is found in soil and the intestines of healthy humans and animals (Murayama et al. 1995; Nakanishi and Tanaka 2010). It has the ability to survive at lower pH, relatively higher bile concentrations and temperature compared with *Lactobacillus* and *Bifidobacterium* and has been used in a wide range of human and veterinary intestinal diseases as one important symbiotic bacteria (Yang et al. 2010, 2012). Previous studies have demonstrated that *C. butyricum* could significantly increase intramuscular fat in the breast muscle without affecting abdominal fat in broilers (Zhao et al. 2013); however, the mechanism for this phenomenon is not clear. Therefore, the objective of this study was to assess the possibility that *C. butyricum* could target intestinal microbiota, which suppressed the *ANGPTL4*.
level and, as a consequence, positively affect intramuscular fat accumulation by decreasing the fatty acid oxidation in the breast muscle of broilers.

Materials and methods

Experimental design, animals and management

A total of 192 one-day-old male Arbor Acres broiler chicks with similar body weight (BW) (43.23 ± 0.21 g) were obtained from a commercial hatchery (Jiangsu Jinghai Poultry Industry Group Co. Ltd., Haimen, Jiangsu, China). The broilers were randomly allocated into 12 wire mesh cages that were then randomly divided into 2 groups (treatments, 6 cages per treatment). Each cage had 13,000 (130 × 100) cm² of floor space and was equipped with two nipple drinkers and one feeder. The dietary treatments were corn-soybean meal-based diets supplemented with 0 and 1 × 10⁹ cfu (2 g) of C. butyricum/kg of diet (denoted as Control and CB, respectively). C. butyricum was provided by Shandong Baolai-leeal Biotechnology Co. Ltd., Tai’an, Shandong, China. Previous research conducted in our laboratory (Poultry Institute, Chinese Academy of Agricultural Sciences, Yangzhou, Jiangsu, China) showed that supplementation with C. butyricum at a level of 1 × 10⁹ cfu/kg of diet enhanced growth performance and intramuscular fat deposition (Zhao et al. 2017). Therefore, 1 × 10⁹ cfu/kg of C. butyricum was used in the diets. The strain of C. butyricum used in this study was C. butyricum Cb-2 (CCTCC M 2011384). All of the experimental diets were in mash form and were formulated to meet or slightly exceed the nutrient requirements recommended by the National Research Council (1994). The diet compositions are shown in Table 1. The C. butyricum powder was first mixed with the premix and subsequently mixed with other ingredients; the mix was stored in covered containers prior to feeding.

The 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 next two 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 of the birds were vaccinated with Newcastle disease and infectious bronchitis vaccines (Pulike Biological engineering Inc., Luoyang, He’nan, China) on days 7 and 21 and with the infectious bursal disease vaccine (Pulike Biological engineering Inc.) on days 14 and 28. The birds were fed ad libitum and had free access to water throughout the entire experiment.

Sample collection

Blood samples were taken from the wing vein of 6 birds (1 bird per cage) from each treatment on the morning of days 21 and 42 of the experiment (after the birds were fasted for 12 h with free access to water) using sterilised needles and non-heparinised tubes. The blood samples were incubated at 37 °C for 2 h and were then centrifuged at 1500 × 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 for blood sampling were fed ad libitum, had free access to water for approximately 2 h and were then slaughtered by exsanguination under deep sodium pentobarbitone anaesthesia (30 mg/kg BW, i.v.). Tissue samples were obtained from the ileum.
and breast muscle. Some of the breast muscle samples (1.0 cm × 1.0 cm × 1.5 cm) 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 ileum (0.3 cm) and breast muscle (0.3 cm × 0.3 cm × 0.5 cm) samples were immediately stored in RNAfixer (RP1302, BioTeke Co. Ltd, Beijing, China), preserved at 4°C overnight and transferred to −20°C for subsequent extraction of total RNA. The caecal contents were placed in sterilised tubes and quickly frozen in liquid nitrogen and then stored at −80°C for subsequent DNA extraction. Abdominal fat and breast muscle samples were collected as described by Yang et al. (2010). Abdominal fat was calculated as the percentage of abdominal fat weight relative to BW (Cai et al. 2009). The intramuscular fat content was determined by extraction with diethyl ether in a Soxhlet apparatus and expressed as the permillage of fresh muscle weight.

**Determination of serum hormone levels and biochemical parameters**

The insulin (INS), growth hormone (GH) and leptin (LEP) levels in the serum were determined using commercial RIA kits obtained from the Beijing North Institute of Biological Technology (Beijing, China) according to the manufacturer’s protocol.

The concentrations of total cholesterol (TC) and triglyceride (TG) in the serum were measured by colorimetric enzymatic methods using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The concentration of very low-density lipoprotein (VLDL) in the serum was measured by a specific ELISA kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions.

**Determination of serum angiopoietin-like protein 4**

The concentration of ANGPTL4 in the serum was measured by a specific ELISA kit (178073, US Biological Life Sciences, Swampscott, MA) according to the manufacturer’s instructions.

**Enzyme activity assays**

The breast muscle samples were assayed for enzymatic activity of lipoprotein lipase (LPL, EC 3.1.1.34) and hormone-sensitive lipase (HSL, EC 3.1.1.3) using the same procedures as described by Zhao et al. (2013).

**Real-time quantitative PCR analysis of gene expression**

The real-time quantitative PCR analysis of gene expression in the breast muscle and ileum was performed using the same procedures as described by Zhao et al. (2013). The gene-specific primers for adipocyte fatty acid-binding protein (A-FABP), carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), long-chain acyl-CoA dehydrogenase (LCAD), LPL, ANGPTL4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal reference gene. Relative mRNA expression levels of each target gene were normalised to the control using the $2^{-\Delta\Delta CT}$ method (Li et al. 2015).

**Real-time PCR quantification of the predominant bacterial divisions**

The DNA of the caecal contents was extracted using QIAMP DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The DNA concentration and purity were determined by

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

| Gene name  | Genebank number | Primers position | Primers sequences (5′−3′) | Product size (bp) | Reference |
|-----------|-----------------|------------------|---------------------------|----------------|-----------|
| A-FABP    | NM_204290       | Forward          | AAGACTGCTACCTGGGCTGTA     | 104             | This study |
|           |                 | Reverse          | CCCACCCACGCTTTCTGATA      |                 |           |
| CPT1      | DQ314726        | Forward          | GATGTTGACCTCAACCCGCT      | 137             | This study |
|           |                 | Reverse          | GAGTTCGGGCGGCT           |                 |           |
| CPT2      | NM_001031287    | Forward          | AACTCCGTAGCCCATAGCGCG    | 109             | This study |
|           |                 | Reverse          | GCAAGATTAGGTGGTTACGGTGA   | 203             | Wang et al. (2012) |
| LCAD      | NM_001006511    | Forward          | GCTGCGGCTCTGTTGGCTAC     | 97              | Huang et al. (2015) |
|           |                 | Reverse          | CTTAGGGGAGCGGCAAGGC       |                 |           |
| LPL       | NM_205282       | Forward          | AACTGAAAGACCGTGCTCAG      | 164             | This study |
| ANGPTL4   | XM_001232283    | Forward          | GCCTGCGGCTCTGTTGGCTAC    | 164             | This study |
| GAPDH     | NM_204305       | Forward          | GTGAAAGTCGAGTAACCGG       | 108             | Drayan et al. (2007) |
|           |                 | Reverse          | CAGTAGGATGGTGGTGGCTGC    |                 |           |

*ANGPTL4: angiopoietin-like protein 4; A-FABP: adipocyte fatty acid-binding protein; CPT1: carnitine palmitoyltransferase 1; CPT2: carnitine palmitoyltransferase 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LCAD: long-chain acyl-CoA dehydrogenase; LPL: lipoprotein lipase.*
measuring the absorbance at 260 and 280 nm (NanoDrop ND-2000c, Thermo Fisher Scientific, Wilmington, DE). Real-time quantitative PCR assay was performed and subjected to the same conditions used for tissue samples. The amplifying primer sets of Firmicutes, Bacteroidetes and total bacteria are listed in Table 3. The abundance of Firmicutes and Bacteroidetes was expressed as a proportion of total bacterial 16S rDNA according to the equation: relative quantification = $2^{\frac{-1}{Ct\ target-Ct\ total\ bacteria}}$, where Ct represents the threshold cycle (Chen et al. 2008; Mao et al. 2010; Zhao et al. 2013).

### Statistical analysis

The experiment was conducted as a completely randomised design. The data are expressed as the mean ± standard error of the mean (SEM). The significance of differences between treatments was tested by independent samples t-tests (Statistical Analysis System 8.1 software, SAS Institute, Inc., Cary, NC). The cage was considered the experimental unit. 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.

### Results

#### Growth performance and fat deposition

All of the broilers appeared to be healthy throughout the entire experimental period (data not shown). Supplementation of *Clostridium butyricum* increased ($p < 0.05$) ADG and tended ($0.05 < p < 0.10$) to increase ADFI during the grower phase and throughout the entire period of the experiment (Table 4). Addition of *C. butyricum* reduced ($p = 0.047$) FCR during the grower phase and tended ($p = 0.091$) to decrease FCR throughout the entire experimental period. In addition, supplementation with *C. butyricum* increased ($p = 0.008$) the breast muscle intramuscular fat content at 42 days of age and tended ($p = 0.056$) to enhance the breast muscle intramuscular fat content at 21 days of age. However, the abdominal fat at both 21 and 42 days of age was not significantly affected by the addition of *C. butyricum*.

#### Serum hormone levels and serum biochemical parameters

The birds in the *C. butyricum*-supplemented groups showed higher ($p = 0.032$) INS levels in the serum at 21

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### 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, GGAGYATGTTAATTCGAAGCA 126 | Guo et al. (2008)                |
|                | Firm1060R, AGCTGACCACACATGCGAC |                                  |           |
| **Bacteroidetes** | Bact934F, GGARCATGTGGTTAATTCGAATGAT | 126 | Guo et al. (2008) |
|                | Bact1060R, AGCTGACGACACCACATGCAG |                                  |           |
| **Total bacteria** | Eub338F, ACCTCTACGGGAGGCAGCAG 200 | Fierer et al. (2005)            |
|                | Eub518R, ATTACCGCGGCTGCTGG     |                                  |           |

### Table 4. Effect of *Clostridium butyricum* on growth performance and fat deposition.

| Item                                | Control (mean ± SEM) | CB (mean ± SEM) | $p$ value |
|-------------------------------------|----------------------|-----------------|-----------|
| 1 to 21 days*                        | 41.31 ± 0.47         | 41.65 ± 0.40    | .598      |
| Average daily feed intake, g/day    | 26.72 ± 0.22         | 27.19 ± 0.35    | .281      |
| Feed conversion rate, g/g           | 1.55 ± 0.02          | 1.53 ± 0.01     | .602      |
| 22 to 42 days*                      | 133.86 ± 2.24        | 139.79 ± 2.30   | .095      |
| Average daily feed intake, g/day    | 71.75 ± 1.52         | 77.92 ± 1.83*   | .027      |
| Feed conversion rate, g/g           | 1.87 ± 0.02*         | 1.80 ± 0.02     | .047      |
| 1 to 42 days*                       | 87.58 ± 1.11         | 90.72 ± 1.10    | .073      |
| Average daily gain, g/day           | 49.23 ± 0.73*        | 52.55 ± 0.93*   | .016      |
| Feed conversion rate, g/g           | 1.71 ± 0.02          | 1.67 ± 0.01     | .091      |
| 21 days*                            | Abdominal fat, (%)   | 0.50 ± 0.05     | .709      |
| Intradamuscular fat (breast muscle), mg/g | 3.39 ± 0.38        | 4.58 ± 0.38     | .056      |
| 42 days*                            | Abdominal fat, (%)   | 1.37 ± 0.18     | .294      |
| Intradamuscular fat (breast muscle), mg/g | 4.96 ± 0.60        | 7.73 ± 0.54*    | .008      |

*a,b Means within a row with different letters differ significantly ($p < 0.05$).

c Control = basal diet.

cb = the basal diet supplemented with $1 \times 10^9$ cfu of *Clostridium butyricum* kg.

Data are the means for 6 replicates of 16 chicks per cage.

Data are the means for 6 replicates of 1 chick per cage.
days of age and lower (p < .05) GH levels in the serum at both 21 and 42 days of age (Table 5). However, the LEP, TC, TG and VLDL levels in the serum at both 21 and 42 days of age were not significantly affected by the addition of \( C.\ butyricum \).

**Angiopoietin-like protein 4 level**

The ANGPTL4 mRNA expression in the ileum and ANGPTL4 concentration in the serum of broilers at 21 days of age was not significantly affected by the addition of \( C.\ butyricum \) (Figure 1(a,c)). However, supplementation with \( C.\ butyricum \) decreased (\( p < .05 \)) the ANGPTL4 mRNA expression in the ileum and ANGPTL4 concentration in the serum of broilers at 42 days of age (Figure 1(b,d)).

**Lipid metabolism related enzyme activities**

The activities of LPL and HSL in the breast muscle at 21 days of age were not significantly affected by the addition of \( C.\ butyricum \) (Figure 2(a,c)). However, supplementation with \( C.\ butyricum \) increased (\( p = .017 \)) the activity of LPL, but it reduced (\( p = .025 \)) the activity of HSL in the breast muscle at 42 days of age (Figure 2(b,d)).

**Lipid metabolism gene expression**

The A-FABP, CPT1, CPT2, LCAD and LPL mRNA levels in the breast muscle at 21 days of age were not significantly affected by the addition of \( C.\ butyricum \) (Figure 3(a)). However, birds supplemented with \( C.\ butyricum \) had lower (\( p < .05 \)) CPT1, CPT2 and LCAD mRNA levels and higher (\( p = .044 \)) LPL mRNA levels in the breast muscle at 42 days of age (Figure 3(b)).

**Quantitation of caecal microbiota**

The relative abundance of \( \text{Firmicutes} \) in the caecal contents at 21 days of age and the relative abundance of \( \text{Bacteroidetes} \), \( \text{Firmicutes/Bacteroidetes} \) ratio in the caecal contents at both 21 and 42 days of age were not significantly affected by the addition of \( C.\ butyricum \) (Figure 4(a–c)). However, the birds in the \( C.\ butyricum \)-supplemented group showed a higher (\( p = .020 \)) relative abundance of \( \text{Firmicutes} \) in the caecal contents at 42 days of age (Figure 4(b)).

**Discussion**

**Effect of \( C.\ butyricum \) on growth performance and fat deposition of broilers**

In recent years, achieving higher growth performance and better body composition, including higher levels of intramuscular fat and lower levels of abdominal fat, has gained increasing interest. The results of this study showed that: the ADG and breast muscle intramuscular fat content increased over the 42 day experimental period; the ADFI tended to increase during the grower phase and throughout the entire period of the experiment; and the FCR decreased during the grower phase and tended to decrease throughout the entire experimental period. However, abdominal fat was not affected at either 21 or 42 days of age by \( C.\ butyricum \) supplementation at a level of \( 1 \times 10^9 \) cfu/kg of diet. These data demonstrated that \( C.\ butyricum \) supplemented at the level of \( 1 \times 10^9 \) cfu/kg improved growth performance and body composition of broilers. Liao, Ma, et al. (2015) and Liao, Wu, et al. (2015) reported that supplementation with \( C.\ butyricum \) at a level of \( 1 \times 10^9 \) cfu/kg enhanced ADG during the starter phase and decreased abdominal fat at 42 days of age.
without affecting ADFI or FCR in either phase of the experiment. Zhao et al. (2013) observed that diets containing $1 \times 10^9$ cfu/kg of *C. butyricum* increased the ADFI, ADG and breast muscle intramuscular fat contents over a 42-day experimental period, but the FCR and abdominal fat were not affected. Yang et al. (2010) reported that supplementation with *C. butyricum* at dietary levels of $1 \times 10^9$ cfu/kg did not affect abdominal fat, but markedly increased the breast muscle intramuscular fat content of broilers at 42 days of age. However, Zhang et al. (2011a) observed no effect on growth performance in broilers when *C. butyricum* was fed at a level of $1 \times 10^9$ cfu/kg. The discrepancy among these studies may be attributed to several factors, such as broiler breeds, diet compositions and administration levels. The enhanced growth performance and breast muscle intramuscular fat content of the broilers in the *C. butyricum*-supplemented group 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 (Zhang et al. 2011a; Yang et al. 2012).

**Effect of *C. butyricum* on the serum hormones levels and serum biochemical parameters of broilers** 

Fat deposition is regulated by a variety of hormones (Chilliard 1993; Huang et al. 2006; Miao et al. 2008). Numerous studies established that the levels of INS, GH and LEP in the serum are the three main indicators that reflect the lipid metabolism of animal (Chung et al. 1983; Dunshea et al. 1992; Etherton 2000; Louveau and Gondret 2004; Ramsay 2004; Sun et al. 2006). Insulin, which regulates the rate of glucose conversion to lipids, is generally considered to be related to the rate of lipogenesis and lipolysis (Chung et al. 1983; Dunshea et al. 1992). Growth hormone can contribute to an increase in adenylate cyclase and lipase activities, thereby leading to an increase in lipolysis (Zhao 2014). Leptin is a paracrine-functioning hormone.
produced by adipose tissue that can affect feed intake and energy metabolism and subsequently acts on tissue to alter lipid accretion by altering lipolysis and lipogenesis (Zhao et al. 2013). Hence, the higher INS and lower GH levels in the group supplemented with *C. butyricum* in this study indicated the lower capacity of the broilers to degrade fat. The observed effects of *C. butyricum* supplementation on enhancing breast

![Figure 2](image-url) Lipid metabolism related enzyme activities in the breast muscle of broilers fed diets with or without *Clostridium butyricum* (*C. butyricum*) (1 x 10^9 cfu/kg) supplementation. (a) Lipoprotein lipase (LPL) activity in the breast muscle of broilers at 21 days of age. (b) LPL activity in the breast muscle of broilers at 42 days of age. (c) Hormone-sensitive lipase (HSL) activity in the breast muscle of broilers at 21 days of age. (d) HSL activity in the breast muscle of broilers at 42 days of age. The value of each treatment is the mean of 6 replicates of 1 chick per cage, and the vertical bar represents standard error of the mean. Means with different letters differ significantly (p < .05).

![Figure 3](image-url) Adipocyte fatty acid-binding protein (A-FABP), carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), long-chain acyl-CoA dehydrogenase (LCAD), and lipoprotein lipase (LPL) mRNA expression in the breast muscle of broilers fed diets with or without *Clostridium butyricum* (*C. butyricum*) (1 x 10^9 cfu/kg) supplementation at 21 (a) and 42 (b) days of age. The value of each treatment is the mean of 6 replicates of 1 chick per cage, and the vertical bar represents standard error of the mean. Means with different letters differ significantly (p < .05).
muscle intramuscular fat content in this study may be partially attributed to the increased INS levels, reduced GH levels and unchanged LEP levels in the serum produced by *Clostridium butyricum*.

The concentrations of serum lipids and lipoproteins are indicative of metabolic regulations at steady state, especially of the basal adjustment of fatty acid circulation between the adipose tissue and the liver (Mossab et al. 2002). Zhao et al. (2013) reported that supplementation of *Clostridium butyricum* at a dietary level of $1 \times 10^9$ cfu/kg did not affect the TC and TG levels 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. The homeostatic mechanism between the lipogenesis by liver and the uptake and utilisation of fatty acid by peripheral tissues such as muscle of broilers may play an important role in these results.

**Effect of *C. butyricum* on angiopoietin-like protein 4 level of broilers**

Angiopoietin-like protein 4 is proposed to be a circulating mediator between intestinal microbiota and the lipid metabolism of the host. Greiner and Bäckhed (2011) reported that the gut microbiota can suppress the intestinal expression of ANGPTL4, which not only alleviates LPL inhibition and promotes LPL-mediated TG storage but also reduces fatty acid oxidation in skeletal muscle. Hence, it is well recognised that ANGPTL4 is an indicator that could reflect the effects of *Clostridium butyricum* on lipid metabolism. The lower ANGPTL4 mRNA expression in the ileum and ANGPTL4 concentration in the serum of broilers at 42 days of age in the group supplemented with *Clostridium butyricum* in this study indicated the lower inhibition of LPL and lower capacity of lipodieresis in broiler breast muscle. This finding is consistent with the *Clostridium butyricum* effects on breast muscle intramuscular fat content obtained in this study, suggesting that the increased breast muscle intramuscular fat content was partially attributed to the decreased ANGPTL4 mRNA expression in the ileum and ANGPTL4 concentrations in the serum produced by *Clostridium butyricum*.

**Effect of *C. butyricum* on lipid metabolism related enzyme activities and gene expression of broilers**

In birds, it is important to study the process of lipid uptake and lipodieresis of skeletal muscle in order to explain the molecular mechanism of *Clostridium butyricum* on increasing breast muscle intramuscular fat content of broilers. Lipoprotein lipase catalyses the hydrolysis of TG from circulating CM and VLDL, which is a rate-limiting step in the lipid transport into peripheral tissues (Cai et al. 2009). Voshol et al. (2001) reported that a high expression of muscle LPL was associated with increased intramuscular TG accumulation. Hence, the higher LPL activity and mRNA levels in the breast muscle in the group supplemented with *Clostridium butyricum* in this study indicated the greater capacity of the broilers to accumulate intramuscular fat in the breast muscle. Adipocyte fatty acid-binding protein is a cytosolic protein, which plays an important role in the fatty acid uptake in muscle (Kim et al. 2004; Ye et al. 2010). The unchanged A-FABP mRNA expression in the breast muscle in the group supplemented with *Clostridium butyricum* in this study indicated that supplementation with

![Figure 4](image-url)

*Figure 4.* Cecal microbiota of broilers fed diets with or without *Clostridium butyricum* (*C. butyricum*) ($1 \times 10^9$ cfu/kg) supplementation. (a) *Firmicutes* and *Bacteroidetes* relative abundance in the caecal contents of broilers at 21 days of age. (b) *Firmicutes* and *Bacteroidetes* relative abundance in the caecal contents of broilers at 42 days of age. (c) *Firmicutes*/*Bacteroidetes* ratio in the caecal contents of broilers at 21 and 42 days of age. The value of each treatment is the mean of 6 replicates of 1 chick per cage, and the vertical bar represents standard error of the mean. Means with different letters differ significantly ($p < .05$).
C. butyricum at the level of 1 × 10^9 cfu/kg of diet didn’t alter the lipid uptake in the breast muscle. Hormone-sensitive lipase is the key enzyme catalysing the hydrolysis of stored TG in adipose tissue into free fatty acid and glycerol (Huang et al. 2006). Fatty acid β-oxidation occurs primarily in the mitochondria, where long-chain fatty acids have to cross the mitochondrial membranes through the CPT system, and CPT1 is generally considered to be a rate-limiting enzyme of the fatty acid oxidation (Bartlett and Eaton 2004; Motoki et al. 2012). Long-chain acyl-CoA dehydrogenase, which functions as a rate-limiting enzyme of β-oxidation, catalyses the next step in the lipodieresis pathway. Therefore, it is well recognised that HSL, CPT1, CPT2 and LCAD are four main indicators that reflect lipodieresis. The lower activity of HSL and the mRNA levels of CPT1, CPT2 and LCAD in the breast muscle in the group supplemented with C. butyricum in this study indicated the lower capacity of the broilers to degrade fat. The quantity of intramuscular fat in the breast muscle is the net outcome of lipid uptake and degradation. Therefore, the increased intramuscular fat content in the breast muscle of broilers fed C. butyricum is likely associated with the unchanged lipid uptake and decreased lipodieresis. All of these findings are consistent with the effects of C. butyricum on ANGPTL4 levels obtained in this study, suggesting that the increased LPL activity and mRNA levels and decreased lipodieresis in the breast muscle was partially attributed to the decreased ANGPTL4 mRNA expression in the ileum and ANGPTL4 concentration in the serum produced by C. butyricum.

Information on the effects of C. butyricum on lipid metabolism related enzyme activities and gene expression in the breast muscle of broilers is lacking. Zhang et al. (2011b) observed that diets containing 1 × 10^9 cfu/kg of C. butyricum increased the LPL activity in the breast muscle of broilers at 42 days of age. Zhao et al. (2013) reported that supplementation with C. butyricum at the level of 1 × 10^9 cfu/kg of diet increased the LPL activity and mRNA levels in the breast muscle of broilers at 42 days of age. It appeared that the LPL activity and mRNA levels in the breast muscle response observed in this study was similar to those of broilers in previous studies.

**Effect of C. butyricum on quantitation of caecal microbiota of broilers**

In chickens, the caecum is the main site of fermentation in the gastrointestinal tract. It harbours a diverse microbial community, and *Firmicutes* and *Bacteroidetes* are the two dominant bacterial phyla in the broiler caecum (Zhu et al. 2002; Lu et al. 2003; Corrigan et al. 2011). The increased *Firmicutes* relative abundance in the caecal contents of C. butyricum-supplemented broilers at 42 d of age in this study indicated that supplementation with C. butyricum at the level of 1 × 10^9 cfu/kg of diet alters the microbiota in the caecum. The increased acetic acid, butyric acid, valeric acid, and total short-chain fatty acids in the caecal contents and the reduced pH of caecal contents by C. butyricum supplementation have favourable effects on caecal microorganisms (Zhang et al. 2011a), which may have partially contributed to the increased *Firmicutes* relative abundance in the caecal contents. Previous studies have demonstrated that the *Firmicutes* relative abundance and *Firmicutes/Bacteroidetes* ratio in the gut had a positive correlation with the fat deposition, but the *Bacteroidetes* relative abundance in the gut had a negative correlation with the fat deposition (Ley et al. 2005, 2006; Turnbaugh et al. 2006; Guo et al. 2008; Zhao 2014). Additionally, recent data have revealed that the gut microbiota, especially *Firmicutes* and *Bacteroidetes*, contributes to host metabolism by several mechanisms including changed energy harvested from the diet, altered endocrine function, and modulation of lipid metabolism (Greiner and Bäckhed 2011). Therefore, the observed effects of C. butyricum supplementation on improving growth performance and breast muscle intramuscular fat content, enhancing serum insulin levels, decreasing serum GH levels, decreasing ANGPTL4 mRNA expression in the ileum and ANGPTL4 concentration in the serum, reducing fatty acid oxidation related enzyme activities and gene expression in the breast muscle may be partially attributed to the increased relative abundance of *Firmicutes* in the caecal contents by C. butyricum.

**Conclusions**

In conclusion, supplementation with C. butyricum at a level of 1 × 10^9 cfu/kg in diets facilitated intramuscular fat accumulation in the breast muscle of broilers at 42 days of age, and the enhanced breast muscle intramuscular fat content by C. butyricum supplementation may be partially attributed to the increased relative abundance of *Firmicutes*, which suppressed the ANGPTL4 level and, as a consequence, positively affected the lipid metabolism by increasing the insulin levels in the serum, enhancing the LPL activity and mRNA levels in the breast muscle, reducing the GH levels in the serum, and decreasing the fatty acid oxidation related enzyme activities and gene expression in the breast muscle. However, more researches are needed to determine the direct relationship between
the relative abundance of *Firmicutes* and ANGPTL4 expression, and whether one or more genera among the *Firmicutes* may be biomarkers of intramuscular fat accumulation in the future studies.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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