Acetate, Propionate and Butyrate Reduce Appetite and Fat Accumulation in Mice via Modulating Relevant Genes and Hormones

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Research

Keywords: acetate, propionate, butyrate, appetite, fat accumulation, mice

DOI: https://doi.org/10.21203/rs.3.rs-41561/v1
Abstract

Acetate, propionate and butyrate, three of the most common short chain fatty acids (SCFAs), can be produced when some non-digestible carbohydrates enter the large intestine and undergo bacterial fermentation. This study was designed to investigate the effects of these three SCFAs on appetite regulation and lipid metabolism, and to what extent appetite contributed to the beneficial influences of SCFAs. In a 35-day study, a total of 48 C57BL/6 male mice were randomly allocated into six groups: (1) control; (2) 5% sodium acetate; (3) 5% sodium propionate; (4) 5% sodium butyrate; (5) pair fed 1; (6) pair fed 2. The results showed that sodium acetate reduced serum triglyceride, free fatty acids, glucose and interleukin (IL) 6 levels ($P < 0.05$), increased serum glucagon-like peptide 1 and leptin levels ($P < 0.05$), down-regulated the mRNA expressions of fatty acid synthase, peroxisome proliferator activated receptor and lipoprotein lipase ($P < 0.05$), and up-regulated the mRNA expressions of fasting induced adipose factor, nuclear respiratory factor 1, mitochondrial transcription factor A, tumor necrosis factor receptor superfamily member 9, cytochrome c oxidase IV and free fatty acid receptor 2 ($P < 0.05$). Sodium propionate also reduced serum IL-1β level ($P < 0.05$), increased serum peptide YY level ($P < 0.05$), down-regulated the mRNA expressions of acetyl-CoA carboxylase and sterol regulatory element binding protein 1c ($P < 0.05$), and up-regulated the mRNA expression of transmembrane protein 26 ($P < 0.05$). Besides, sodium butyrate decreased average daily feed intake ($P < 0.05$), down-regulated the mRNA expression of myosin heavy-chain ($MyHC$) $b$ ($P < 0.05$), and up-regulated the mRNA expressions of lipase hormone-sensitive, $MyHC$ $a$ and carnitine palmitoyltransferase-1 $a$ ($P < 0.05$). Moreover, the metabolic benefits of SCFAs were partly attributed to the reduction of feed intake. Taken together, SCFAs could reduce appetite and fat accumulation via modulating relevant genes and hormones, which might further illustrate the potential mechanisms that underlay the impacts of SCFAs on lipid homeostasis and body weight control.

Introduction

Obesity, one of the most severe health problems that contemporary people are faced with, has inevitably drawn our great attention. Due to the imbalance between energy intake and expenditure, a lot of complex symptoms called metabolic syndrome are likely to occur [1]. It increases the risk of metabolic diseases like type 2 diabetes and cardiovascular disease [1]. Currently, more and more studies have suggested dietary treatment as one of the most efficient strategies to control obesity level. Among them, dietary fiber has been associated with suppressed appetite, reduced body weight gain, and improved postprandial glucose response [2, 3]. Thus, there has been a refocus on the investigation of dietary fiber and its potential mechanisms.

Dietary fiber passes through the small intestine without being influenced by digestive enzymes [4]. However, it can be catabolized by bacteria in the hindgut [5]. And the main products of bacterial intestinal fermentation are short chain fatty acids (SCFAs), with acetate, propionate and butyrate as the most abundant ones, in the approximate ratio of 60:20:20 [6]. It has been suggested that the effects of dietary fiber on metabolism, to a certain degree, are mediated by SCFAs and their receptors, namely free fatty acid receptor 2 and free fatty acid receptor 3 [7]. Previous studies demonstrated that acetate could be
used for *de novo* synthesis of lipid and propionate was classically regarded as a gluconeogenic substrate [8]. Moreover, butyrate was a main energy source for both hosts and cells [9]. Despite all of these, recent studies showed that SCFAs could act as signaling molecules to be involved in several physiological process, including reductions in insulin resistance and appetite, thus contributing to glucose homeostasis and body weight control [10, 11]. Besides, propionate and butyrate activated intestinal gluconeogenesis, which was necessary for the metabolic benefits generated by SCFAs or dietary fiber [12]. Thus, more studies are needed badly to investigate this controversy and the specific roles of acetate, propionate and butyrate, respectively.

Therefore, in this study, we determined the effects of different SCFAs on the appetite regulation and lipid metabolism of mice, and more importantly, to what extent appetite contributed to the beneficial influences of SCFAs.

**Methods And Materials**

**Animal, management and diet**

Experimental procedure and animal care were accomplished in accordance with the guide for the care and use of laboratory animals provided by the institutional animal care advisory committee for Sichuan Agricultural University. All animal protocols used in this study were approved by the animal care and use committee of Sichuan Agricultural University under permit number DKY-B20131704.

In the present study, a total of 56 C57BL/6 male mice (aged 4-week, purchased from Chengdu Dashuo Experimental Animal Co, Ltd) were randomly allocated to 7 groups (n = 8): (1) control; (2) 5% sodium acetate; (3) 5% sodium propionate; (4) 5% sodium butyrate; (5) pair fed 1; (6) pair fed 2; (7) pair fed 3. All of the mice received a high fat diet (D12492) without (control and pair fed) or with 5% (w/w) sodium acetate, propionate and butyrate, respectively. Soy oil and lard were used as fat sources for the high fat diet. Sodium acetate, propionate and butyrate were purchased from Sigma. As sodium acetate, propionate and butyrate were supposed to reduce feed intake, three pair fed groups received the same amount of high fat diet as those of sodium acetate, propionate and butyrate, respectively. All of the mice were individually caged under a 12 hour light and 12 hour dark cycle, with free access to water. The whole experiment lasted for 5 weeks.

**Growth Performance**

The body weight of each mice was measured every week. And the feed intake was recorded every day. The average daily body weight gain (ADG), average daily feed intake (ADFI) and the ratio of feed to gain (F/G) were calculated based on the values mentioned above.

**Slaughter And Sample Collection**
For the ADFI of sodium acetate and sodium propionate was almost the same, so at the end of the experiment, control group, SCFAs groups, and two pair fed groups were sacrificed. At 8:00 on day 36, after overnight fasting and ether anesthesia, blood samples were collected by heart puncture, centrifuged at 3000 × g, and stored at -20°C. All of the mice were sacrificed according to previously described methods [13]. Epididymal fat, gastrocnemius and liver were obtained and stored at -80°C for further analyses.

Biochemical Analyses

The triglyceride (TG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-c), low density lipoprotein-cholesterol (LDL-c), free fatty acid (FFA), and glucose of serum were detected by commercial assay kits purchased from Nanjing Jiancheng Biochemistry (Nanjing, China) according to the producer's directions. The insulin, leptin, glucagon-like peptide 1 (GLP-1), peptide YY (PYY), adiponectin (ADP), resistin, ghrelin, interleukin (IL) 1β, IL 6, IL 10 and tumour necrosis factor α (TNFα) of serum were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits purchased from Jiangsu Jingmei Biotechnology (Co., Ltd., Yancheng, China) according to the producer's directions.

Rna Isolation And Reverse Transcription

Total RNA was prepared from frozen epididymal fat, gastrocnemius and liver with Trizol Reagent (TaKaRa Biotechnology, Dalian, China) according to the producer's directions. The purity and concentration of total RNA were measured by spectrophotometer (Beckman Coulter DU800), and the ratio of OD260 : OD280 was guaranteed to range from 1.8 to 2.0, which suggested a low degradation level. After reverse transcription with RT Reagents (TaKaRa Biotechnology, Dalian, China), complementary DNA was obtained.

Real-time Quantitative Pcr

After reverse transcription, the mRNA levels of several relevant genes were detected by real-time quantitative PCR with SYBR Premix Ex Taq reagents (TaKaRa Biotechnology, Dalian, China) and CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories, Richmond, CA) according to previously described methods [14]. All primers presented in Table 1 [15] were commercially purchased from TaKaRa Biotechnology (Dalian, China). They included fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), peroxisome proliferator activated receptor (PPAR), sterol regulatory element binding protein 1c (SREBP-1c), lipoprotein lipase (LPL), carnitine palmitoyltransferase-1α (CPT-1α), lipase hormone- sensitive (LIPE), free fatty acid receptor 2 (FFAR2), free fatty acid receptor 3 (FFAR3), fasting induced adipose factor (Fiaf), PPARγ coactivator-1α (PGC-1α), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (Tfam), b subunit of the mitochondrial H+-ATP synthase (β-F1-ATPase), cytochrome c oxidase IV (COX IV), cytochrome c somatic (Cyt-c), myosin heavy-chain (MyHC), transmembrane protein 26 (Tmem26), tumor necrosis factor receptor superfamily member 9 (CD137), T-box1 (Tbx1), Leptin, Adiponectin and
Resistin. The cycling conditions were: first pre-denaturation at 95 °C for 30 s, then 40 cycles at 95 °C for 5 s, next at annealing temperature for 30 s, finally at 72 °C for 60 s. A melting curve analysis was also carried out to verify the purity and specificity of reactions. \( \beta\text{-actin} \) was chosen as the reference gene to normalize the mRNA expressions of target genes, and the relative gene expression compared to reference gene was calculated based on previously described methods [16]. All sample analyses were run repeatedly in triplicate at the same time, and on the same plate. At last, an average one was utilized to calculate the values mentioned above.
Table 1
Primers lists used for real time PCR assay

| Primer       | Forward primer (5’to3’) | Reverse primer (5’to3’) |
|--------------|-------------------------|-------------------------|
| β-actin      | GGGCCAACCCGTGAAAAGATGA  | CAGCCTGGATGGCTACGTACA   |
| FAS          | TGGTGAAATTGCTCCGAAAAGA  | CACGTTCATCAGGAGGCTATG   |
| ACC          | CGAAGGGGTTACATTGCCTA    | GGATGTTCCCTCTGTTTGA     |
| SREBP-1c     | GCATGCCATGGGCAAGTAC     | CCACATAGATCTCGGAGTGGT   |
| PPAR         | ATGTCTCACAATGCCATCAGGTT | GCTCGCAGATCAGCAGACTCT   |
| LIPE         | ATGCCACTCACCTCTGATCC    | CTGTCTGCTCTTCCCCTAG     |
| LPL          | TGAAAGCCGGAGAGACTCAG    | AGTGTCAGCCAGACTTTCAG    |
| CPT-1α       | CCCCAATACCCCTACATCCT    | ATCCCCGATACCCCTGTC      |
| Resistin     | CCTGCTAAGTCTCTGCCAC     | GGTCTCATCGATGGGACACA    |
| Aiponectin   | TGACGCACACAAAGGGCTC     | ACCTGCACAAGTTCCCCTTG    |
| Leptin       | TGGCTTTGCTCTATCTGTC     | TCCTGGTACAAGTGGCTTG     |
| Fiaf         | CACCCACTTACAGGCGG       | GAAGTCCACAGAGCGGTCA     |
| PGC-1α       | AGCCGTGACACTGACAACAG    | GCTGCATGGTTCTGAGTCTAAG  |
| Nrf1         | CAAGTCACAGGCTCATGT      | GTTACCTCATCAGCTGCCG     |
| Tfam         | AAGAACGCATGGAGGAGA      | TTCTGGGAGAGTTGCAGTT     |
| β-F1-ATPase  | CGTGAGGGCAATGATTTATACC  | TCCTGGTCTCTGGAATGATTCCAGA |
| COX IV       | TTAAACGAGAGCTTGCGCGAG   | CCAAATCAGAAGAGGCGCAG    |
| Cyt-c        | ATAGGGGACATGTCACCTCAAAC | GTGGATTACGATGACCTGAAAG |
| Tmem26       | ACCCTGTCTACCCACAGAG     | TGTGGTGGGAATGCCTAGG     |
| CD137        | CGTGCAAGAACTCTGTAAGA    | GCACCACCTATGCGAGAAG     |
| Tbx1         | GGCAGGCAGACGAATGTTC     | TTGTCATCTACGGGCACAAAG   |
| MyHC I       | CTTTCTACAGGGCTGGCTTAC   | CTCTTTCACAGACTTCGCA     |

FAS fatty acid synthase; ACC acetyl-CoA carboxylase; SREBP-1c sterol regulatory element binding protein 1c; PPAR peroxisome proliferator activated receptor; LIPE lipase hormone-sensitive; LPL lipoprotein lipase; CPT-1α carnitine palmitoyltransferase-1α; Fiaf fasting induced adipose factor; PGC-1α PPARγ coactivator-1α; Nrf-1 nuclear respiratory factor 1; Tfam mitochondrial transcription factor A; β-F1-ATPase b subunit of the mitochondrial H+ -ATP synthase; COX IV cytochrome c oxidase IV; Cyt-c cytochrome c somatic; Tmem26 transmembrane protein 26; CD137 tumor necrosis factor receptor superfamily member 9; Tbx1, T-box1; MyHC myosin heavy-chain; FFAR free fatty acid receptor.
| Primer | Forward primer (5'to3') | Reverse primer (5'to3') |
|--------|-------------------------|------------------------|
| MyHC Ila | TTCCAGAAGCCTAAGGTGGTC | GCCAGCCAGTGATGTTGTAAT |
| MyHC Ilx | CAACCCATACGACTACGCCT | CATCAGAAGTGAGCCAGAAAT |
| MyHC IIb | CTTGTCTGACTCAAGCCTGCC | TCGCTCCTTTTCAGACTTCCG |
| FFAR2 | ACAGTGGAGGGGACCAAGAT | GGGGACTCTCTACTCGGTGA |
| FFAR3 | TTGCTAAACCTGACCATTTCGG | GATAGGCCACGCTCAGAAAAC |

FAS fatty acid synthase; ACC acetyl-CoA carboxylase; SREBP-1c sterol regulatory element binding protein 1c; PPAR peroxisome proliferator activated receptor; LIPE lipase hormone-sensitive; LPL lipoprotein lipase; CPT-1α carnitine palmitoyltransferase-1α; Fiaf fasting induced adipose factor; PGC-1α PPARγ coactivator-1α; NRF-1 nuclear respiratory factor 1; Tfam mitochondrial transcription factor A; β-F1-ATPase b subunit of the mitochondrial H+ -ATP synthase; COX IV cytochrome c oxidase IV; Cyt-c cytochrome c somatic; Tmem26 transmembrane protein 26; CD137 tumor necrosis factor receptor superfamily member 9; Tbx1, T-box1; MyHC myosin heavy-chain; FFAR free fatty acid receptor.

**Statistical analysis**

Descriptive statistic program was performed to assess whether data was normally distributed by using SPSS 20.0 (Statistical Product and Service Solutions, Inc, USA). Then one-way ANOVA test was performed to compare the difference of normally distributed data among groups, followed by Duncan’s multiple-range test. Results were shown as mean and SEM. $P < 0.05$ was considered statistically significant. And, $P < 0.1$ was considered a tendency.

**Results**

**Growth performance**

As shown in Table 2, sodium butyrate significantly reduced the ADFI of C57BL/6 mice compared with the control group ($P < 0.05$). However, no significant differences were observed among the six groups regarding ADG and F/G ($P > 0.05$).
Table 2
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the growth performance of C57BL/6 mice

|                   | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|-------------------|---------|---------|------------|----------|------------|------------|-----|---------|
| Initial BW g      | 11.71   | 11.72   | 11.72      | 11.72    | 11.71      | 11.71      | 0.13| 0.999   |
| Final BW g        | 18.91   | 17.45   | 17.08      | 17.61    | 17.60      | 18.18      | 0.27| 0.442   |
| ADG g             | 0.21    | 0.16    | 0.15       | 0.17     | 0.17       | 0.18       | 0.01| 0.377   |
| ADFI g            | 3.21a   | 3.08ab  | 3.06ab     | 2.79b    | 3.07ab     | 2.78b      | 0.05| 0.023   |
| F/G               | 16.24   | 19.63   | 23.89      | 18.44    | 19.85      | 15.69      | 0.98| 0.177   |

BW body weight; ADFI average daily feed intake; ADG average daily gain; F/G the ratio of feed to gain;

Within a row, means without a common superscript differ (P< 0.05).

Table 3
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the epididymal fat weight and perinephric fat weight of C57BL/6 mice

|                   | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|-------------------|---------|---------|------------|----------|------------|------------|-----|---------|
| Epididymal fat g  | 0.17ab  | 0.15ab  | 0.12b      | 0.15ab   | 0.16ab     | 0.19a      | 0.01| 0.055   |
| Perinephric fat g | 0.01    | 0.01    | 0.01       | 0.01     | 0.01       | 0.01       | 0.00| 0.230   |

Within a row, means without a common superscript differ (P< 0.05).

Serum Metabolites

According to Table 4, sodium acetate and butyrate significantly reduced the serum TG level of C57BL/6 mice compared with the control group (P< 0.05). Sodium acetate also significantly reduced the serum FFA level of C57BL/6 mice compared with the control group (P< 0.05). Besides, sodium SCFAs significantly reduced the serum glucose level of C57BL/6 mice compared with the control group (P< 0.05). In addition, the serum TG level of sodium butyrate group was significantly lower than that of pair fed 2 group (P< 0.05). The serum FFA level of sodium acetate group was significantly lower than that of pair fed 1 group (P< 0.05). However, no significant differences were observed among the six groups regarding TC, HDL-c and LDL-c (P> 0.05).
Table 4
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the serum metabolites of C57BL/6 mice

|                    | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM  | \( P \)-value |
|--------------------|---------|---------|------------|----------|------------|------------|------|---------------|
| TG mmol/L          | 1.21\(^a\) | 0.92\(^{bc}\) | 0.98\(^{abc}\) | 0.81\(^c\) | 1.06\(^{abc}\) | 1.22\(^{ab}\) | 0.04 | 0.044         |
| TC mmol/L          | 4.60    | 4.43    | 4.41       | 4.48     | 4.52       | 4.47       | 0.04 | 0.826         |
| HDL-c mmol/L       | 1.66    | 1.79    | 1.71       | 1.67     | 1.58       | 1.52       | 0.04 | 0.355         |
| LDL-c mmol/L       | 0.89    | 0.80    | 0.76       | 0.85     | 0.79       | 0.81       | 0.02 | 0.635         |
| FFA mmol/L         | 0.29\(^a\) | 0.16\(^b\) | 0.23\(^{ab}\) | 0.23\(^{ab}\) | 0.29\(^a\) | 0.26\(^a\) | 0.01 | 0.025         |
| Glucose mmol/L     | 3.72\(^a\) | 3.09\(^b\) | 3.11\(^b\) | 3.19\(^b\) | 3.10\(^b\) | 3.19\(^b\) | 0.06 | 0.029         |

TG triglyceride; TC total cholesterol; HDL-c high density lipoprotein-cholesterol; LDL-c low density lipoprotein-cholesterol; FFA free fatty acids;

\(^{a-c}\)Within a row, means without a common superscript differ \((P<0.05)\).

**Serum Hormones**

As shown in Table 5, sodium acetate and propionate significantly increased the serum GLP-1 and leptin levels of C57BL/6 mice compared with the control group \((P<0.05)\). Sodium propionate and butyrate also significantly increased the serum PYY level of C57BL/6 mice compared with the control group \((P<0.05)\). In addition, the serum GLP-1 and leptin levels of sodium acetate and propionate groups were significantly higher than those of pair fed 1 group \((P<0.05)\). The serum GLP-1 and PYY levels of sodium butyrate group were significantly higher than those of pair fed 2 group \((P<0.05)\). However, no significant differences were observed among the six groups regarding adiponectin, resistin, ghrelin and insulin \((P>0.05)\).
Table 5
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the serum hormones of C57BL/6 mice

|                | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|----------------|---------|---------|------------|----------|------------|------------|------|---------|
| GLP-1 pmol/L   | 0.35<sup>bc</sup> | 0.46<sup>a</sup> | 0.46<sup>a</sup> | 0.44<sup>ab</sup> | 0.32<sup>c</sup> | 0.30<sup>c</sup> | 0.02 | 0.002   |
| PYY pg/mL      | 32.15<sup>b</sup> | 33.59<sup>ab</sup> | 37.54<sup>a</sup> | 38.85<sup>a</sup> | 29.31<sup>b</sup> | 31.45<sup>b</sup> | 0.85 | 0.003   |
| Adiponectin µg/L| 10.86   | 10.30   | 10.91      | 10.04    | 10.35      | 10.79      | 0.26 | 0.912   |
| Resistin µg/L  | 4.43    | 4.22    | 4.10       | 3.39     | 4.11       | 4.23       | 0.13 | 0.256   |
| Ghrelin ng/L   | 40.35   | 40.07   | 39.15      | 40.39    | 41.17      | 40.34      | 1.39 | 0.999   |
| Insulin mIU/L  | 1.01    | 0.92    | 0.95       | 0.97     | 0.98       | 0.99       | 0.02 | 0.816   |
| Leptin pg/mL   | 84.53<sup>b</sup> | 100.55<sup>a</sup> | 105.96<sup>a</sup> | 84.91<sup>b</sup> | 83.31<sup>b</sup> | 78.13<sup>b</sup> | 2.47 | 0.020   |

GLP-1 glucagon-like peptide 1; PYY peptide YY;

<sup>a-c</sup>Within a row, means without a common superscript differ (P < 0.05).

**Serum Cytokines**

According to Table 6, sodium propionate and butyrate significantly reduced the serum IL-1β level of C57BL/6 mice compared with the control group (P < 0.05). Sodium acetate also significantly reduced the serum IL-6 level of C57BL/6 mice compared with the control group (P < 0.05). In addition, the serum IL-6 level of sodium acetate group was significantly lower than that of pair fed 1 group (P < 0.05). However, no significant differences were observed among the six groups regarding IL-10 and TNF-α (P > 0.05).
Table 6
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the serum cytokines of C57BL/6 mice

|                | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM  | P-value |
|----------------|---------|---------|------------|----------|------------|------------|------|---------|
| IL-1β ng/L     | 11.38^a | 10.83^ab| 9.98^bc    | 9.68^bc  | 9.79^bc    | 9.29^c     | 0.20 | 0.013   |
| IL-6 pg/mL     | 36.44^a | 32.01^b | 33.53^ab   | 35.85^a  | 36.36^a    | 36.44^a    | 0.49 | 0.023   |
| IL-10 pg/mL    | 210.63  | 209.12  | 219.76     | 229.78   | 208.16     | 207.28     | 3.24 | 0.281   |
| TNF-α pg/mL    | 36.97   | 31.83   | 35.10      | 41.77    | 40.14      | 39.56      | 1.13 | 0.107   |

IL interleukin; TNFα tumour necrosis factor α;

^a–cWithin a row, means without a common superscript differ (P < 0.05).

The Mrna Expressions Of Related Genes In Epididymal Fat

As shown in Table 7, sodium acetate and propionate significantly up-regulated the mRNA expression of Leptin in epididymal fat of C57BL/6 mice compared with the control group (P < 0.05). Sodium acetate and propionate also significantly down-regulated the mRNA expression of FAS in epididymal fat of C57BL/6 mice compared with the control group (P < 0.05). Besides, sodium acetate significantly up-regulated the mRNA expression of Fiaf in epididymal fat of C57BL/6 mice compared with the control group (P < 0.05). And sodium SCFAs significantly down-regulated the mRNA expression of LPL in epididymal fat of C57BL/6 mice compared with the control group (P < 0.05). However, no significant differences were observed among the six groups regarding Adiponectin, Resistin, ACC, SREBP-1c, PPAR, LIPE and CPT-1α (P > 0.05).
### Table 7
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the lipid metabolism of epididymal fat in C57BL/6 mice

|                  | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|------------------|---------|---------|------------|----------|------------|------------|-----|---------|
| Leptin           | 1.00c   | 1.60ab  | 1.97a      | 1.28bc   | 1.16bc     | 1.13bc     | 0.08| 0.002   |
| Adiponectin      | 1.00    | 1.18    | 0.95       | 1.03     | 1.15       | 1.06       | 0.05| 0.828   |
| Resistin         | 1.00    | 0.74    | 0.95       | 1.09     | 0.93       | 0.85       | 0.05| 0.320   |
| FAS              | 1.00a   | 0.73bc  | 0.59c      | 0.88ab   | 0.88ab     | 0.84ab     | 0.04| 0.014   |
| ACC              | 1.00    | 0.97    | 0.76       | 0.81     | 0.99       | 0.87       | 0.05| 0.581   |
| SREBP-1c         | 1.00    | 1.04    | 0.89       | 0.91     | 0.87       | 0.98       | 0.04| 0.718   |
| PPAR             | 1.00    | 0.93    | 0.90       | 0.81     | 0.84       | 0.69       | 0.03| 0.130   |
| LIPE             | 1.00    | 0.69    | 0.86       | 0.84     | 0.87       | 0.95       | 0.05| 0.549   |
| Fiaf             | 1.00bc  | 1.44a   | 1.41ab     | 1.42ab   | 0.91c      | 0.93c      | 0.07| 0.018   |
| LPL              | 1.00a   | 0.57c   | 0.69bc     | 0.72bc   | 0.87ab     | 0.82abc    | 0.04| 0.016   |
| CPT-1α           | 1.00    | 1.09    | 1.05       | 0.97     | 1.03       | 0.99       | 0.06| 0.994   |

FAS fatty acid synthase; ACC acetyl-CoA carboxylase; SREBP-1c sterol regulatory element binding protein 1c; PPAR peroxisome proliferator activated receptor; LIPE lipase hormone-sensitive; LPL lipoprotein lipase; CPT-1α carnitine palmitoyltransferase-1α; Fiaf fasting induced adipose factor;

*a−c* Within a row, means without a common superscript differ (P < 0.05).

According to Table 8, sodium SCFAs significantly up-regulated the mRNA expressions of *PGC-1α, NRF-1* and *Tfam* in epididymal fat of C57BL/6 mice compared with the control group (P < 0.05). However, no significant differences were observed among the six groups regarding *β-F1-ATPase, COX IV* and *Cyt-c* (P > 0.05).
Table 8
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the mitochondrial biogenesis of epididymal fat in C57BL/6 mice

|                  | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | p-value |
|------------------|---------|---------|------------|----------|------------|------------|-----|---------|
| **PGC-1α**       | 1.00b   | 1.40a   | 1.42a      | 1.36a    | 0.97b      | 0.86b      | 0.06| 0.005   |
| **NRF-1**        | 1.00c   | 1.43ab  | 1.66a      | 1.45ab   | 1.18bc     | 1.11bc     | 0.07| 0.027   |
| **Tfam**         | 1.00c   | 1.58ab  | 1.75a      | 1.60ab   | 1.29abc    | 1.19bc     | 0.07| 0.020   |
| **β-F1-ATPase**  | 1.00    | 1.01    | 1.08       | 0.90     | 0.99       | 0.95       | 0.03| 0.806   |
| **COX IV**       | 1.00    | 1.01    | 1.27       | 1.04     | 1.24       | 1.03       | 0.07| 0.757   |
| **Cyt-c**        | 1.00    | 1.18    | 1.34       | 1.17     | 1.03       | 1.40       | 0.09| 0.752   |

PGC-1α PPARγ coactivator-1α; NRF-1 nuclear respiratory factor 1; Tfam mitochondrial transcription factor A; β-F1-ATPase b subunit of the mitochondrial H+ -ATP synthase; COX IV cytochrome c oxidase IV; Cyt-c cytochrome c somatic;

a-c Within a row, means without a common superscript differ (P< 0.05).

As shown in Table 9, sodium propionate and butyrate significantly up-regulated the mRNA expression of Tmem26 in epididymal fat of C57BL/6 mice compared with the control group (P< 0.05). Sodium acetate and propionate also significantly up-regulated the mRNA expression of CD137 in epididymal fat of C57BL/6 mice compared with the control group (P< 0.05). However, no significant differences were observed among the six groups regarding TBX-1 (P> 0.05).

Table 9
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the beige adipocyte differentiation of epididymal fat in C57BL/6 mice

|                  | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | p-value |
|------------------|---------|---------|------------|----------|------------|------------|-----|---------|
| **Tmem26**       | 1.00bc  | 1.35ab  | 1.46a      | 1.49a    | 0.93c      | 0.87c      | 0.06| 0.002   |
| **CD137**        | 1.00c   | 1.52ab  | 1.65a      | 1.41abc  | 1.14bc     | 1.17bc     | 0.07| 0.026   |
| **Tbx-1**        | 1.00    | 1.05    | 1.01       | 1.11     | 1.12       | 1.11       | 0.05| 0.969   |

Tmem26 transmembrane protein 26; CD137 tumor necrosis factor receptor superfamily member 9; Tbx1, T-box1;

a-c Within a row, means without a common superscript differ (P< 0.05).
The Mrna Expressions Of Related Genes In Gastrocnemius Muscle

According to Table 10, sodium acetate significantly down-regulated the mRNA expression of PPAR in gastrocnemius of C57BL/6 mice compared with the control group (P < 0.05). Sodium butyrate also significantly up-regulated the mRNA expression of LIPE in gastrocnemius of C57BL/6 mice compared with the control group (P < 0.05). However, no significant differences were observed among the six groups regarding FAS, ACC, SREBP-1c, LPL and CPT-1α (P > 0.05).

Table 10
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the lipid metabolism of gastrocnemius muscle in C57BL/6 mice

|          | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM  | P-value |
|----------|---------|---------|------------|----------|------------|------------|------|---------|
| FAS      | 1.00    | 1.02    | 1.07       | 0.91     | 1.15       | 1.17       | 0.04 | 0.509   |
| ACC      | 1.00    | 0.95    | 1.16       | 0.92     | 1.13       | 1.04       | 0.03 | 0.261   |
| SREBP-1c | 1.00    | 1.07    | 1.16       | 0.97     | 1.02       | 1.12       | 0.03 | 0.454   |
| PPAR     | 1.00    | 0.73    | 0.88       | 0.92     | 1.12       | 1.11       | 0.04 | 0.014   |
| LIPE     | 1.00    | 1.18    | 1.22       | 1.61     | 1.17       | 1.12       | 0.05 | 0.015   |
| LPL      | 1.00    | 0.90    | 1.06       | 0.94     | 1.02       | 0.91       | 0.03 | 0.706   |
| CPT-1α   | 1.00    | 0.99    | 1.17       | 0.94     | 0.99       | 1.09       | 0.04 | 0.485   |

FAS fatty acid synthase; ACC acetyl-CoA carboxylase; SREBP-1c sterol regulatory element binding protein 1c; PPAR peroxisome proliferator activated receptor; LIPE lipase hormone-sensitive; LPL lipoprotein lipase; CPT-1α carnitine palmitoyltransferase-1α;

Within a row, means without a common superscript differ (P < 0.05).

As shown in Table 11, sodium SCFAs significantly up-regulated the mRNA expressions of PGC-1α and COX IV in gastrocnemius of C57BL/6 mice compared with the control group (P < 0.05). Sodium propionate and butyrate also significantly up-regulated the mRNA expression of NRF-1 in gastrocnemius of C57BL/6 mice compared with the control group (P < 0.05). Sodium acetate and propionate significantly up-regulated the mRNA expression of Tfam in gastrocnemius of C57BL/6 mice compared with the control group (P < 0.05). However, no significant differences were observed among the six groups regarding β-F1-ATPase and Cyt-c (P > 0.05).
Table 11
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the mitochondrial biogenesis of gastrocnemius muscle in C57BL/6 mice

|                | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|----------------|---------|---------|------------|----------|------------|------------|-----|---------|
| PGC-1α         | 1.00b   | 2.07a   | 2.17a      | 1.70a    | 0.97b      | 0.94b      | 0.11| 0.001   |
| NRF-1          | 1.00c   | 1.31abc | 1.38ab     | 1.40a    | 1.02bc     | 0.99c      | 0.05| 0.032   |
| Tfam           | 1.00c   | 1.90ab  | 2.17a      | 1.70abc  | 1.07c      | 1.25bc     | 0.11| 0.004   |
| β-F1-ATPase    | 1.00    | 0.99    | 1.20       | 0.92     | 1.19       | 1.04       | 0.04| 0.136   |
| COX IV         | 1.00b   | 1.63a   | 1.59a      | 1.58a    | 1.01b      | 1.22ab     | 0.08| 0.025   |
| Cyt-c          | 1.00    | 1.11    | 0.98       | 0.83     | 0.99       | 0.97       | 0.04| 0.540   |

PGC-1α PPARγ coactivator-1α; NRF-1 nuclear respiratory factor 1; Tfam mitochondrial transcription factor A; β-F1-ATPase β subunit of the mitochondrial H+ -ATP synthase; COX IV cytochrome c oxidase IV; Cyt-c cytochrome c somatic;

\( {\text{a-cWithin a row, means without a common superscript differ (} P < 0.05\text{).}} \)

According to Table 12, sodium butyrate significantly up-regulated the mRNA expression of MyHC \( {\text{a}} \), and down-regulated the mRNA expression of MyHC \( {\text{b}} \) in gastrocnemius of C57BL/6 mice compared with the control group \( (P < 0.05) \). However, no significant differences were observed among the six groups regarding MyHC \( {\text{b}} \) and MyHC \( {\text{x}} \) \( (P > 0.05) \).

Table 12
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the myosin heavy-chain (MyHC) isoformse of gastrocnemius muscle in C57BL/6 mice

|               | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|---------------|---------|---------|------------|----------|------------|------------|-----|---------|
| MyHC \( {\text{a}} \) | 1.00    | 1.05    | 1.09       | 1.05     | 1.20       | 1.13       | 0.04| 0.781   |
| MyHC \( {\text{a}} \) | 1.00b   | 1.08b   | 1.27ab     | 1.56a    | 1.07b      | 1.16b      | 0.05| 0.013   |
| MyHC \( {\text{b}} \) | 1.00a   | 1.06a   | 1.16a      | 0.61b    | 1.15a      | 1.05a      | 0.05| 0.002   |
| MyHC \( {\text{x}} \) | 1.00    | 0.94    | 1.24       | 1.15     | 0.91       | 0.84       | 0.05| 0.143   |

MyHC myosin heavy-chain;

\( {\text{a-bWithin a row, means without a common superscript differ (} P < 0.05\text{).}} \)
According to Table 13, sodium propionate significantly down-regulated the mRNA expressions of ACC and SREBP-1c in liver of C57BL/6 mice compared with the control group \( (P<0.05) \). Sodium butyrate also significantly up-regulated the mRNA expression of CPT-1α in liver of C57BL/6 mice compared with the control group \( (P<0.05) \). However, no significant differences were observed among the six groups regarding FAS, PPAR, LIPE and LPL \( (P>0.05) \).

|                      | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | \( P \)-value |
|----------------------|---------|---------|------------|----------|------------|------------|-----|--------------|
| FAS                  | 1.00    | 1.09    | 1.05       | 1.32     | 1.17       | 1.23       | 0.06| 0.720        |
| ACC                  | 1.00<sup>ab</sup> | 0.71<sup>bc</sup> | 0.69<sup>c</sup> | 1.03<sup>a</sup> | 1.07<sup>a</sup> | 1.01<sup>ab</sup> | 0.05| 0.050        |
| SREBP-1c             | 1.00<sup>ab</sup> | 0.71<sup>bc</sup> | 0.64<sup>c</sup> | 0.87<sup>abc</sup> | 1.02<sup>ab</sup> | 1.14<sup>a</sup> | 0.05| 0.038        |
| PPAR                 | 1.00    | 1.12    | 0.94       | 1.24     | 1.09       | 1.14       | 0.05| 0.484        |
| LIPE                 | 1.00    | 1.24    | 0.93       | 1.30     | 1.06       | 1.16       | 0.05| 0.211        |
| LPL                  | 1.00    | 1.10    | 0.94       | 1.30     | 1.20       | 1.21       | 0.06| 0.487        |
| CPT-1α               | 1.00<sup>b</sup> | 1.06<sup>b</sup> | 0.94<sup>b</sup> | 1.82<sup>a</sup> | 1.12<sup>b</sup> | 1.22<sup>b</sup> | 0.08| 0.011        |

FAS fatty acid synthase; ACC acetyl-CoA carboxylase; SREBP-1c sterol regulatory element binding protein 1c; PPAR peroxisome proliferator activated receptor; LIPE lipase hormone-sensitive; LPL lipoprotein lipase; CPT-1α carnitine palmitoyltransferase-1α;

\( a^-c \) Within a row, means without a common superscript differ \( (P<0.05) \).

As shown in Table 14, sodium propionate and butyrate significantly up-regulated the mRNA expressions of PGC-1α in liver of C57BL/6 mice compared with the control group \( (P<0.05) \). However, no significant differences were observed among the six groups regarding NRF-1, Tfam,β-F1-ATPase, COX IV and Cyt-c \( (P>0.05) \).
Table 14
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the mitochondrial biogenesis of liver in C57BL/6 mice

|            | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM | P-value |
|------------|---------|---------|------------|----------|------------|------------|-----|---------|
| **PGC-1α** | 1.00b   | 1.68abc | 1.80ac     | 1.91a    | 1.21bc     | 1.15bc     | 0.11| 0.047   |
| **NRF-1**  | 1.00    | 1.53    | 1.60       | 1.30     | 1.17       | 1.10       | 0.08| 0.138   |
| **Tfam**   | 1.00    | 1.34    | 1.33       | 1.17     | 1.11       | 1.04       | 0.08| 0.729   |
| **β-F1-ATPase** | 1.00 | 1.20    | 1.01       | 1.32     | 1.05       | 1.15       | 0.07| 0.752   |
| **COX IV** | 1.00    | 0.96    | 0.96       | 1.18     | 0.92       | 0.92       | 0.05| 0.696   |
| **Cyt-c**  | 1.00    | 0.89    | 1.05       | 0.97     | 0.92       | 1.05       | 0.06| 0.969   |

PGC-1α, PPARγ coactivator-1α; NRF-1 nuclear respiratory factor 1; Tfam mitochondrial transcription factor A; β-F1-ATPase b subunit of the mitochondrial H+ -ATP synthase; COX IV cytochrome c oxidase IV; Cyt-c cytochrome c somatic

Within a row, means without a common superscript differ (P< 0.05).

The Mrna Expressions Of Ffar2 And Ffar3

According to Table 15, sodium propionate and butyrate significantly up-regulated the mRNA expression of FFAR3 in epididymal fat of C57BL/6 mice compared with the control group (P< 0.05). Sodium SCFAs also significantly up-regulated the mRNA expression of FFAR2 in epididymal fat of C57BL/6 mice compared with the control group (P< 0.05). In addition, the mRNA expression of FFAR2 of sodium SCFAs groups was significantly higher than that of pair fed groups (P< 0.05). However, no significant differences were observed among the six groups regarding FFAR2 and FFAR3 in gastrocnemius muscle and liver (P> 0.05).
Table 15
Effects of dietary sodium acetate, sodium propionate and sodium butyrate supplementation on the free fatty acid receptors in C57BL/6 mice

|                     | Control | Acetate | Propionate | Butyrate | Pair fed 1 | Pair fed 2 | SEM  | P-value |
|---------------------|---------|---------|------------|----------|------------|------------|------|---------|
| Epididymal fat      |         |         |            |          |            |            |      |         |
| **FFAR3**           | 1.00c   | 1.54abc | 1.64ab     | 1.74a    | 1.19abc    | 1.10bc     | 0.09 | 0.046   |
| **FFAR2**           | 1.00c   | 1.35ab  | 1.40a      | 1.34ab   | 0.91c      | 0.85c      | 0.06 | 0.002   |
| Gastrocnemius muscle|         |         |            |          |            |            |      |         |
| **FFAR3**           | 1.00    | 1.03    | 1.25       | 0.97     | 1.02       | 1.03       | 0.04 | 0.348   |
| **FFAR2**           | 1.00    | 1.08    | 1.43       | 1.15     | 1.17       | 1.16       | 0.04 | 0.109   |
| Liver               |         |         |            |          |            |            |      |         |
| **FFAR3**           | 1.00    | 1.09    | 1.44       | 1.40     | 1.00       | 1.01       | 0.07 | 0.215   |
| **FFAR2**           | 1.00    | 1.13    | 1.21       | 1.36     | 1.16       | 1.15       | 0.06 | 0.661   |

a−c: Within a row, means without a common superscript differ (P< 0.05).

Discussion

The prevalence of obesity has increased rapidly over last decades, and the roles of gut microorganisms and SCFAs in metabolic homeostasis have been highlighted. Previous studies showed that SCFAs could reduce appetite via the gut-brain neural circuit [17]. According to our study, sodium butyrate significantly reduced the ADFI of C57BL/6 mice. Besides, sodium SCFAs increased the concentrations of GLP-1, PYY and/or leptin in serum. GLP-1 and PYY are secreted by L cells, and respond closely and quickly to feed intake [18, 19]. Administrations of these gut hormones resulted in enhanced satiety and reduced energy intake, which were considered as good strategies to combat obesity [20, 21]. Leptin is produced primarily by white adipose tissue and involved in many physiological processes like feeding behaviour and metabolic status [22]. Leptin signalling failure was associated with hyperphagia while an infusion of it could reduce feed intake and increase energy expenditure [23, 24]. Thus, sodium SCFAs reduced ADFI possibly via regulating GLP-1, PYY and leptin, with sodium acetate having the greatest impacts.

SCFAs contribute greatly to improved glucose homeostasis and insulin sensitivity [25]. They controlled body energy utilization and maintained metabolic status via FFAR2, a sensitive sensor for excessive dietary energy [26]. Previous studies also showed that SCFAs activated intestinal gluconeogenesis, which was crucial for the benefits generated by SCFAs [12]. According to our results, we found that sodium SCFAs reduced serum TG, FFA and/or glucose levels, which were partly consistent with other studies [8, 27]. Besides, reduced postprandial glucose level was often associated with increased GLP-1 and PYY levels [28, 29], thus SCFAs might modulate glucose level via gut-derived hormones. More importantly, in
the light of our study, SCFAs regulated these metabolic parameters mainly or partly by reduced feed intake.

Chronic low-grade inflammation is one of the key factors that result in obesity and its complications, such as non-alcoholic fatty liver disease [30]. IL-6 concentration was associated with circulating lipopolysaccharides level, which was supposed to initiate inflammation-related insulin resistance [31]. And IL-1β was correlative with G protein-coupled receptor 109A, whose signalling was involved in type 2 diabetes [32]. Our results showed that sodium SCFAs reduced the concentrations of IL-6 and/or IL-1β in serum, which indicated that SCFAs could attenuate chronic low-grade inflammation and contribute to insulin sensitivity.

SCFAs could modulate adipogenesis and lipolysis in adipose tissue, skeletal muscle and liver via several mechanisms [25]. FAS is a core enzyme that catalyzes fatty acid synthesis, and ACC as well as its product, malonyl-CoA, can act as building blocks for de novo fatty acid synthesis [33, 34]. SCFAs regulated these gene expressions through activating AMP activated protein kinase (AMPK) [27]. Besides, SREBP-1c enhances the transcription of targeted genes that encode the enzymes of cholesterol biosynthesis and uptake [35]. Consistent with previous studies [36], we found that SCFAs reduced the mRNA expressions of FAS, ACC and/or SREBP-1c, which could attenuate adipogenesis and cholesterol synthesis. Moreover, suppression of Fiaf increased LPL activity in adipocytes, thereby increasing triglyceride storage [37]. Our study demonstrated that sodium SCFAs up-regulated the mRNA expression of Fiaf while down-regulated the mRNA expression of LPL, which indicated a reduction of triglyceride storage in adipose tissue. In addition, LIPE is the chief enzyme responsible for FFA mobilization and CPT-1α participates in fatty acid oxidation and catalyzes the very first step [38, 39]. According to our study, SCFAs enhanced the mRNA expressions of LIPE and/or CPT-1α, thus promoting lipolysis and fatty acid oxidation.

Impairment of mitochondrial function is associated with diabetes due to the fact that reduced ATP synthesis rate is often observed before decreased glucose tolerance [40]. PGC-1α acts as a crucial transcriptional coactivator of both nuclear and non-nuclear receptor transcription factors involved in the energy metabolism of cells, such as PPAR and NRFs [41]. Also, NRFs regulate Tfam, a nuclear factor that activates mitochondria replication and transcription [42]. Moreover, mitochondrial cytochrome c oxidase, known as complex IV, catalyzes nitrite reduction under anaerobiosis [42]. Therefore, our results found that SCFAs improved mitochondrial function by enhancing the mRNA expressions of PGC-1α, NRF-1, Tfam and/or COX IV, which further contributed to glucose regulation and body weight control. Besides, generally, muscles with a high proportion of fast-twitch fiber (MyHC-IIb) are relatively poor in mitochondrial activity [43]. Our studies showed that sodium butyrate enhanced MyHC IIa mRNA level while reduced MyHC-IIb level in gastrocnemius, suggesting an improvement of muscle mitochondrial function.

Typically, adipocytes could be divided into two types, namely white fat cells and brown fat cells. White fat cells store energy while brown fat cells produce heat and combat obesity and diabetes [44]. Specially,
brown fat could dissipate chemical energy as heat by utilizing mitochondrial contents [44]. Apart from classical brown fat, some brown-like cells called beige cells are derived from white adipose, and they are also characterized by strong antiobesity properties [45]. When animals are under cold circumstances or given chronic β-adrenergic stimulation, beige adipocytes will experience a kind of phenotypic transdifferentiation, then browning will occur morphologically and histochemically [44]. Our study found that some beige adipocyte markers, such as *Tmem26* and/or *CD137*, were elevated by sodium SCFAs, indicating a promotion in beige adipogenesis.

*FFAR 2* and *FFAR 3*, also called G-protein-coupled receptor 43 and 41, could be bound and activated by SCFAs. They are widely expressed in both small and large intestine [46]. Previous study demonstrated that SCFAs attenuated obesity induced by high fat diet via these receptors [15]. Our results showed that sodium SCFAs only increased the mRNA expressions of FFAR 2 and FFAR 3 in epididymal fat, but not in gastrocnemius muscle or liver, suggesting more direct or indirect influences of SCFAs on these tissues could be investigated further in the future.

**Conclusion**

In summary, our study found that sodium SCFAs could suppress appetite and attenuate fat deposition via modulating related genes and hormones involved in adipogenesis, lipolysis, mitochondrial function and beige adipogenesis. More importantly, SCFAs regulated some metabolic processes mainly or partly by reduced feed intake. These all provided some new insights into the roles of SCFAs in obesity and nonalcoholic fatty liver.

**Declarations**

**Acknowledgements**

The authors sincerely thank Jin Wan for his kind help during the whole study.

**Funding**

This study was financially funded by the National Natural Science Foundation of China (31672436), the earmarked fund for the China Agricultural Research System (CARS-35), Sichuan Province Science and Technology Support Project (2016NYZ0052), the National Basic Research Program of China (2013CB531406), and the National High Technology Research and Development Program of China (2014AA022209).

**Availability of date and materials**

All data generated or analyzed during the study are included in this published article.

**Authors’ contributions**
DWC and XBM designed the whole experiment. ARJ performed the experiment, including chemical analysis, statistical analysis and manuscript writing. BY, JH, HY and JY verified the validity of the experiment and checked the results. PZ, JQL, QYW, HFW and YHL participated in the experiment design and gave valuable advice. All of the authors have read and approved the final version of this manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

All experimental procedures and animal care were accomplished in accordance with the Guide for the Care and Use of Laboratory Animals provided by the Institutional Animal Care Advisory Committee for Sichuan Agricultural University. All animal protocols used in this study were approved by the Animal Care and Use Committee of Sichuan Agricultural University under permit number DKY-B20131704.

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