Bromochloromethane (BCM), an inhibitor of methanogenesis, has been used in animal production. However, little is known about its impact on the intestinal microbiota and metabolic patterns. The present study aimed to investigate the effect of BCM on the colonic bacterial community and metabolism by establishing a Wistar rat model. Twenty male Wistar rats were randomly divided into two groups (control and treated with BCM) and raised for 6 weeks. Bacterial fermentation products in the cecum were determined, and colonic methanogens and sulfate-reducing bacteria (SRB) were quantified. The colonic microbiota was analyzed by pyrosequencing of the 16S rRNA genes, and metabolites were profiled by gas chromatography and mass spectrometry. The results showed that BCM did not affect body weight and feed intake, but it did significantly change the intestinal metabolic profiles. Cecal protein fermentation was enhanced by BCM, as methylamine, putrescine, phenylethylamine, tyramine, and skatole were significantly increased. Colonic fatty acid and carbohydrate concentrations were significantly decreased, indicating the perturbation of lipid and carbohydrate metabolism by BCM. BCM treatment decreased the abundance of methanogen populations, while SRB were increased in the colon. BCM did not affect the total colonic bacterial counts but significantly altered the bacterial community composition by decreasing the abundance of actinobacteria, acidobacteria, and proteobacteria. The results demonstrated that BCM treatment significantly altered the microbiotic and metabolite profiles in the intestines, which may provide further information on the use of BCM in animal production.

Bromochloromethane (BCM) (CH₂BrCl, CAS no. 74-97-5) is an analog of dihalogenated methane. Unintentional consumption of this unregulated halomethane as a by-product of disinfection in drinking water is one of the major sources for BCM consumption of this unregulated halomethane as a by-product of dis-
have reported the impact of BCM on the other metabolic processes in the GIT, let alone the host whole-body metabolism. Therefore, we hypothesized that oral supplementation of BCM in a monogastric animal with the level per body weight equivalent to that used with ruminants would affect the intestinal microbiota and its metabolic profile. To test this hypothesis, the present study integrated colonic microbiome and metabolomics, coupled with an analysis of bacterial fermentation products to investigate the change in bacterial community and host metabolites in the Wistar rat model. This may provide fundamental information toward the impact of BCM ingestion on GIT bacterial composition and metabolism and on animal production.

**MATERIALS AND METHODS**

**Animals and diets.** The experiment was conducted in compliance with the Chinese regulations concerning the protection of experimental animals, according to the protocol approved by the ethics committee of Nanjing Agricultural University, Nanjing, China.

Adult male Wistar specific-pathogen-free (SPF) rats (n = 20), weighing 200 to 220 g, were housed in stainless steel wire cages on a 12-h reverse light/dark cycle (7:00 a.m. to 7:00 p.m.). The rats were acclimated to the environment for the first week with a standard rodent diet (Table 1). After acclimatization, the rats were randomly allocated to 2 groups of 10 rats each (control and BCM group). All rats were given free access to water and fed the standard diet ad libitum for 6 weeks. For the BCM group, liquid BCM (CAS no. 74-97-5, 99.5% purity; Aladdin, Shanghai, China) was added to the drinking water, according to their drinking volume, to make the final concentration 0.3 mg/l/100 g of BW. The rats were weighed every 2 days. Food residues were recorded daily. At 42 days, each rat was anesthetized between 8:00 a.m. and 11:00 a.m. with diethyl ether. Plasma was collected from the jugular vein before dissection and collection of the luminal contents of the colon and cecum. Colonic tissue samples were collected and immediately snap-frozen in liquid nitrogen.

**Chemical analytical procedures.** The measurement of blood total protein, globulin, albumin, glucose, triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), urea, creatinine, and uric acid was conducted by the enzymatic colorimetric method using an AU2700 autoanalyzer (Olympus, Tokyo, Japan). Plasma concentrations of insulin, glucagon, T3, T4, gastrin, and growth hormone were determined using a radioimmunoassay kit with 125I as a tracer (North Institute of Biological Technology, Beijing, China). The radioactivity of 125I-labeled hormone was measured using an SN-6105 radioimmunoassay gamma counter (Shanghai Hesuo Rhiun Photoelectric Instruments, Shanghai, China). Cecal SCFA content was measured by gas chromatography (GC), as described previously (21).

**Analysis of amino acid-derived metabolites.** The free ammonia concentration of cecal contents was determined by the phenol-hypochlorite method, as described previously (22). Cecal biogenic amines (23) and phenolic and indolic compounds (24) were determined by high-performance liquid chromatography (HPLC), as described previously. Sulfide concentration was determined by the methylene blue method, according to Siegel (25).

**Gas chromatography and mass spectrometry analysis.** The colon contents were weighed and diluted with double-distilled water (1:3 [wt/vol]), and 300 μl of methanol was added to a 100-μl aliquot of supernatant. After incubation for 1 min, 100 μl of supernatant was transferred to a gas chromatography (GC) vial and evaporated using an SPD2010-230 SpeedVac concentrator (Thermo Savant, Holbrook, NY, USA). The dried extract was methoxylated and trimethylsilylated prior to analysis with GC-mass spectrometry (GC-MS), as described previously (26). The metabolites in the gut contents were analyzed using GC-MS (QP2010 Ultra/SE; Shimadzu, Kyoto, Japan). The masses between m/z 50 and 800 were acquired from 7 to 20 min, with a scan speed of 2,500 Hz and event time of 0.30 s. The metabolites were identified in comparison with the database NIST and Wiley. Peak areas were normalized to an internal standard before further analysis. Partial least-squares discriminant analysis (PLS-DA) was calculated using the SIMCA-P 13.0 software (Umetrics, Umeå, Sweden).

**Quantification of methanogens and SRB.** Total bacterial DNA was extracted with 300 mg colonic digesta using the bead-beating and phenol-chloroform extraction methods, as previously described (27). A real-time PCR assay was performed on an ABI 7300 detection system (Applied Biosystems) with ROX reference dye and SYBR fluorescence dye (Takara Bio Inc., Otsu, Japan). The sequences of the selected targets and primers are listed in Table 2.

**Pyrosequencing of colonic bacterial 16S rRNA.** Universal primers (5′-TACGGRAGGCGACGAG-3′ and 5′-AGGTATCTAACTCT-3′) targeting the V3–V4 region of the colonic microbial 16S rRNA gene were chosen for the amplification and subsequent pyrosequencing of the PCR products. After purification with the Agencourt AMPure XP system (Beckman Coulter, USA), the PCR amplicons from different samples were barcoded, pooled to construct the sequencing library, and sequenced on a 454 GS FLX Titanium platform at the Chinese National Human Genome Center (Shanghai, China).

**Bioinformatics analysis.** Raw sequence data generated from pyrosequencing were processed in the mothur version 1.36 software package (28). All sequences of <200 bp, having one or more ambiguous bases, or containing a homopolymer length of ≥8 bp were removed from the data set. Unique sequences were normalized to contain an equal number of sequences in the two groups before the sequences were presumptively identified and aligned against a database of high-quality 16S rRNA bacterial sequences derived from the bacterial Silva database (Silva version 108 [https://www.arb-silva.de/documentation/release-108/]). Using the average neighbor algorithm with a cutoff of 97% identity, these sequences were clustered into operational taxonomic units (OTUs). Representative sequences from each OTU were taxonomically classified with a confidence level of 90% using the RDP classifier (http://rdp.cme.msu.edu/). Alpha diversity was conducted within mothur, namely, Chao1, ACE,

<table>
<thead>
<tr>
<th>Item</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients (%) (as-fed basis)</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>20</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>40.4</td>
</tr>
<tr>
<td>Amylodextrin</td>
<td>13.2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
</tr>
<tr>
<td>Colza oil</td>
<td>7</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Salt</td>
<td>0.2</td>
</tr>
<tr>
<td>Calcium hydrophosphate</td>
<td>1</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>1.3</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Vitamin-mineral mixture</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.5</td>
</tr>
<tr>
<td>Total bacterial DNA</td>
<td>328.8</td>
</tr>
</tbody>
</table>

a DM, dry matter; CHO, carbohydrate; ME, metabolic energy.
b AIN-93G mineral and vitamin mixes.

**TABLE 1** Composition and nutrient concentrations of the experimental diets

**TABLE 2** Chemical composition (g/100 g DM)
TABLE 2 Primers used for quantification of methanogens, SRB, and total bacteria

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer direction</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanogen</td>
<td>mcrA</td>
<td>Forward: TTCGGTGGAATCDCARAGRGC</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GBARGTCTGAWACGTTAGAATCC</td>
<td></td>
</tr>
<tr>
<td>SRB</td>
<td>dsrA</td>
<td>Forward: CCAACATGCAAGGTYTCC</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CGTCAACITGAACCTTAGTGTAG</td>
<td></td>
</tr>
<tr>
<td>Total bacteria</td>
<td></td>
<td>Forward: CGTGTAATAGCTTTCGCG</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGTACCTTGTACGACTT</td>
<td></td>
</tr>
</tbody>
</table>

Shannon, Simpson, and Good’s coverage indices. Significant and unique OTUs in each group were picked using a linear discriminant algorithm (LDA) effect size (i.e., LDA score, >2) (29).

Analysis of bacterial cooccurrence pattern. The bacterial cooccurrence network was constructed as described previously (30). All possible Spearman correlations were calculated between OTUs with more than five sequences. A Spearman correlation coefficient of |r| of >0.9 and a P value of <0.001 were considered to represent a valid cooccurrence event. The nodes in the reconstructed network represent the OTUs at 97% identity, whereas the edges correspond to a strong and significant correlation between nodes. The networks were visualized with Gephi (31).

Statistical analysis. Body weight, feed intake, plasma biochemical parameters (metabolites and hormones), SCFA, amino acid-derived metabolites, and quantitative real-time PCR data were analyzed using the Student t test corrected with the false-discovery rate (FDR). Bacterial abundance at the phylum level was analyzed by the Mann-Whitney test. FDR correction was also used to verify the discriminant metabolites chosen by Wilcoxon-Mann-Whitney P values. The data were expressed as the mean ± standard error of the mean (SEM). A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. All qualified sequences have been deposited in GenBank under accession numbers KM366802 to KM368234.

RESULTS

Effects of BCM treatment on growth performance and biochemical parameters. Body weight at day 42 tended to increase after BCM supplementation (P = 0.07) (Fig. 1A), but feed intake was not affected. BCM supplementation changed body composition and significantly increased spleen weight (see Table S1 in the supplemental material). No differences were observed for liver weight or mesenteric adipose pads. The plasma concentrations of glucose, triglyceride, amino acids, and uric acid were measured to reflect the systemic change in carbohydrate, lipid, protein, and purine metabolism with BCM treatment. Concentrations of glucose and uric acid in the blood were significantly decreased (P < 0.05; see Table S1), while triglyceride levels were slightly decreased in the BCM group compared to those of the control group (P = 0.079; see Table S1) (Fig. 1B).

Effects of BCM treatment on cecal SCFA and amino acid fermentation products. As shown in Fig. 1C, BCM intervention significantly increased the concentrations of butyrate and isovalerate (P < 0.05) and slightly increased the isobutyrate level (P = 0.079), but it did not affect the concentrations of acetate, propionate, and valerate in the cecum. For biogenic amines, the concentrations of cecal methylamine, phenylethylamine, putrescine, and tyramine were significantly increased in the BCM group compared to those in the control group (P < 0.05, Fig. 2A). For phenolic and indolic compounds, skatole concentration was significantly increased (P < 0.05, Fig. 2B), and indole concentration was slightly increased after BCM supplementation (P = 0.082). There was no significant difference in cecal free ammonia concentrations between the two groups. Cecal sulfide production was significantly increased in BCM-treated rats compared to that in the controls (P = 0.023, Fig. 2C).

Effect of BCM treatment on colonic metabolite profile. GC-MS-based metabolomics profiles of the colon content are presented in Fig. S1 in the supplemental material. Fifty metabolites were detected in the colonic content from adult rats. A heatmap analysis based on the Z-value-normalized peak area indicated that the relative concentrations of colonic metabolites decreased in the BCM treatment group, especially for amino acids, carbohydrates, and fatty acid-related compounds. The PLS-DA model also provided good discrimination between the control and BCM groups (see Fig. S2 in the supplemental material). Nine metabolites with an FDR threshold of <0.05 were identified (Table 3). Glucose-6-phosphate, oleic acid, and proline levels were decreased 5.91-fold, 5.31-fold, and 3.69-fold in the BCM group compared to those in the control group. The levels of taurine, hexadecanoic acid, serine, and gluconic acid decreased by half in the BCM group compared to those in the control group. The stearic acid, pyrogallitic acid, glutamic acid, and ornithine levels were 1.5-fold lower in the BCM group than in the control group. The steareic acid, pyrogallitic acid, glutamic acid, and ornithine levels were 1.5-fold lower in the BCM group than in the control group. The affected metabolites were mostly involved in bile acid metabolism (taurine), amino acid metabolism (proline, pyrogallitic acid, glutamic acid, and ornithine), fatty acid biosynthesis (hexadecanoic acid and stearic acid), and glycolysis/glucogenogenesis (glucose-6-phosphate and gluconic acid).

Effect of BCM supplementation on methanogen and SRB populations. Quantitative real-time PCR analysis showed that methyl coenzyme-M reductase (mcrA) gene copies of the methanogens were significantly reduced (P = 0.040; Fig. 1D), while the dissimilatory sulfite reductase α-subunit (dsrA) gene copies of the SRB were increased in the colonic content of the BCM group compared to those in the control group (P = 0.028; Fig. 1D). However, there was no significant difference in total bacteria (as inferred from 16S rRNA gene copy numbers) in the colonic content between the BCM and control groups.

Effect of BCM treatment on colonic bacterial community structure revealed by pyrosequencing. The total number of sequences and OTUs, coverage, bacterial richness, and diversity at a genetic distance of 3% in each colonic sample under different treatments are presented in Table 4. Across all 20 samples, an
average of 8,431 and 7,088 quality sequences from the control and BCM groups, respectively, were classified as bacteria (Table 4). Species richness was increased in the BCM group compared to that in the control group, as reflected by the ACE index with statistical differences ($P < 0.040$; Table 4). Unweighted UniFrac analysis revealed a significant difference between the BCM and control groups (UniFrac score, 0.955; $P < 0.001$).

Pyrosequencing data showed that bacteria belonging to the phyla Firmicutes and Bacteroidetes were the most dominating phylotypes in the control and BCM-supplemented rats (Fig. 3A). No significant changes in the abundance of bacteria belonging to the phyla Bacteroidetes, Firmicutes, and Proteobacteria were observed between the control and BCM groups, whereas BCM supplementation significantly reduced the abundance of bacteria belonging to the phyla Actinobacteria and Acidobacteria. The taxa that were significantly different between the BCM and control groups are shown in Fig. 3. LDA analyses also confirmed that the abundance of Acidobacteria and Actinobacteria at the phylum level was significantly decreased in the BCM group compared to those in the controls (Fig. 3B). Although no significant difference was observed for the abundance of Proteobacteria at the phylum level, bacteria from five different taxa (Oceanospirillales, Novospirillum, Sutterella, Halomonas, and Halomonadaceae) within the Proteobacteria were significantly decreased after BCM supplementation, with most of them detected in the control group only (Fig. 3B).

Cooccurrence patterns of bacteria. The cooccurrence patterns of colonic bacteria in the control and BCM groups are shown

![Figure 1](http://aem.asm.org)
in Fig. 4. Two distinct networks were generated using the OTUs from the control or BCM group, respectively. The cooccurrence network consisted of 359 nodes and 1,169 edges for the control group and 370 nodes and 1,622 edges for the BCM group. The average degree and diameter were 6.51 and 16 in the control group and 8.77 and 22 in the BCM group. Although the modularities of the two networks are similar, bacteria belonging to the phyla Actinobacteria and Proteobacteria, and some from the phylum Bacteroidetes, tended to cluster together and form a major module after BCM ingestion. This module consisted of very diverse bac-

**TABLE 3** Significantly altered metabolites in colon of BCM-supplemented rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Biological role</th>
<th>Metabolic pathway</th>
<th>( P )</th>
<th>FC(^b)</th>
<th>FDR(^c)</th>
<th>VIP(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>Amino acid-derived metabolite</td>
<td>Nitrogen metabolism</td>
<td>0.023</td>
<td>0.65</td>
<td>0.026</td>
<td>1.03</td>
</tr>
<tr>
<td>Ornithine</td>
<td>Amino acid-derived metabolite</td>
<td>Arginine and proline metabolism</td>
<td>0.028</td>
<td>0.65</td>
<td>0.032</td>
<td>1.07</td>
</tr>
<tr>
<td>Proline</td>
<td>Amino acid-derived metabolite</td>
<td>Arginine and proline metabolism</td>
<td>0.016</td>
<td>0.27</td>
<td>0.017</td>
<td>1.07</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
<td>Amino acid-derived metabolite</td>
<td>Glutathione metabolism</td>
<td>0.020</td>
<td>0.71</td>
<td>0.022</td>
<td>1.03</td>
</tr>
<tr>
<td>Taurine</td>
<td>Amino acid-derived metabolite</td>
<td>Primary bile acid biosynthesis</td>
<td>0.003</td>
<td>0.43</td>
<td>0.003</td>
<td>1.60</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>Carbohydrate</td>
<td>Glycolysis and gluconeogenesis</td>
<td>0.014</td>
<td>0.17</td>
<td>0.014</td>
<td>1.17</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>LCFA</td>
<td>Fatty acid biosynthesis</td>
<td>0.005</td>
<td>0.44</td>
<td>0.005</td>
<td>1.74</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>LCFA</td>
<td>Fatty acid biosynthesis</td>
<td>0.031</td>
<td>0.19</td>
<td>0.037</td>
<td>1.81</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>LCFA</td>
<td>Fatty acid biosynthesis</td>
<td>0.014</td>
<td>0.66</td>
<td>0.015</td>
<td>1.40</td>
</tr>
</tbody>
</table>

\(^a\) LCFA, long-chain fatty acids.

\(^b\) FC, fold change. Fold change was calculated as BCM versus control. A value of \(<1.0\) indicates a lower concentration in the BCM group relative to that in the control group.

\(^c\) FDR, false-discovery rate.

\(^d\) Variable importance in the projection (VIP) value was obtained from the PLS-DA model.
terial taxa, with 6 Actinobacteria, 4 Bacteroidetes, 11 Firmicutes, and 11 Proteobacteria (see Table S2 in the supplemental material). The nearest neighbor of each OTU in this module is shown in Table S2.

**DISCUSSION**

BCM has been widely used in ruminants, including cattle (7, 32) and goats (6, 33), because it can effectively inhibit methanogenesis while having no adverse effect on growth performance. As a result, the impact of BCM on archaeal composition has widely been investigated (6, 7, 32, 33). However, there is a paucity of information on the bacterial community and its metabolic pattern after BCM exposure. Given that the GIT is an internal organ with direct contact with BCM ingestion, we hypothesized that this level of BCM ingestion might exert influence on the intestinal microbial community and metabolic profile. Our results showed, similar to findings in previous studies (6–8), that body weight and feed intake of rats were not influenced by BCM supplementation. Moreover, our hypothesis was supported, as the present study also demonstrated that colonic macronutrients, such as amino acids, carbohydrates, and long-chain fatty acids, were significantly decreased, suggesting that the intestinal metabolic profiles were influenced after BCM administration.

Furthermore, results from colonic metabolomics showed that carbohydrates, such as glucose, fructose, glucuronic, and gluconic acid, were decreased after BCM exposure for 6 weeks, indicating

![FIG 3](A) Significantly changed colonic bacteria in BCM-supplemented rats, as revealed by LDA analysis. An LDA score of <2 was considered significant. P, phylum; O, order; F, family; G, genus. (B) Colonic microbiome composition profiles at the phylum level in control and BCM-supplemented rats, as revealed by 16S rRNA sequencing. The values are expressed as the means ± SEM, with 10 rats per group.
that carbohydrate metabolism was disturbed at the local level (Table 3). Glucose-6-phosphate is the intermediate of the glycolysis process in most host cells and bacteria. Decreasing the concentration of colonic glucose-6-phosphate (Table 3) might suggest the inhibition of host glucose metabolism and bacterial glycolysis by BCM. Long-chain fatty acids (LCFA), such as stearic acid, oleic acid, hexadecanoic acid, and linoleic acid, were also decreased in the colon of BCM rats compared to that in rats in the control group (Table 3). It was possible that the oxidation of LCFA was upregulated and thus the source for intracellular lipid biosynthesis increased by BCM. Although no direct evidence for colonic nutrients contributing to the decrease in plasma triglycerides was obtained, the underlying linkage between the parallel decrease in colonic fatty acids and plasma triglycerides may arise from the nutrient transport by mesenteric vessels. A novel finding of the current study was the increase in amino acids in the blood and the decrease in amino acids in the colon (Fig. 2D; see also Fig. S1 in the supplemental material), which indicated that BCM administration might stimulate the amino acid absorption, leading to fewer amino acids in the large intestine. Russell and Martin (34) reported that the inhibition of methanogenesis would reduce amino acid deamination. Nagase and Matsuo (35) also revealed that since methanogen acted as a hydrogen acceptor, the inhibition of methanogenesis by BCM would also inhibit amino acid degradation. The current study results are similar to previous results that amino acids were seldom catabolized after methanogen inhibition by BCM (35). Meanwhile, amino acid absorption and fermentation by bacteria were enhanced (Fig. 1C and 2A and B).

In the present study, the abundance of cecal methanogens, as represented by the number of mcrA gene copies, decreased after BCM treatment for 6 weeks (Fig. 1D), which was in accordance with previous studies that BCM can reduce the abundance of fecal methanogens in rats (8) and ruminal methanogens in cattle (32). SRB, like methanogens, are another group of hydrogenotrophic bacteria. However, it was believed that sulfate reduction and methanogenesis competed against each other (36). In the current study, the abundance of SRB increased and methanogen abundance decreased after BCM treatment (Fig. 1D). SRB are closely associated with various bowel diseases, such as ulcerative colitis, irritable bowel syndrome, and inflammatory bowel disease (37–39). Furthermore, the increase in SRB was coupled with an elevation in sulfide concentration in the feces of BCM rats (Fig. 2C). Sulfide can be produced by the reduction of inorganic sulfur through the sulfate-reducing process. The significant increase in SRB confirmed that the sulfate-reducing process was enhanced. The fermentation of sulfur-containing amino acids (SAA), such as cysteine and methionine, might also release sulfide (40). Research has shown that the fermentation of dietary SAA is the major source of fecal sulfide concentration (41). Thus, the significant increase in cecal sulfide concentration suggests that amino acid fermentation was enhanced. Previous researchers have shown that the increase in hydrogen sulfide can disrupt the gut epithelial tissues and induce DNA damage, and it can even increase the sensitivity of the GIT to virus infection (42,43). Moreover, the reaction of BCM with sulfide compounds may yield a product of greater toxicological significance than hydrogen sulfide (44), which can be detrimental to the GIT.

In the large intestine, undigested protein and other nitrogenous compounds were mainly fermented by bacteria with the production of branched-chain fatty acids (BCFA), amines, and ammonia (45). In the present study, the decrease in colonic amino acids (Table 3), together with the elevation in cecal biogenic amine levels (Fig. 2A), suggested that bacterial decarboxylation of amino acids was stimulated after BCM administration. Methyamine can be released during glycine decarboxylation and the degradation of sarcosine, N,N-methylarginine, adrenaline, choline, and creatine (46–48), which also indicated the enhanced amino acid decarboxylation. Polyamines, such as putrescine, have been shown to exert genotoxic effects on the host and might serve as potential tumor markers (17). High level of amines, especially histamine, tryptam-
ine, β-phenylethylamine, and tyramine, might be toxic to gut health (49). BCFA produced from branched-chain amino acid deamination and microbial fermentation are regarded as indicators of protein fermentation by the large-intestinal bacteria (50). In the present study, the increasing isovalerate and isobutyrate concentrations in the cecum (Fig. 1C) suggested that the BCM can induce incomplete degradation of amino acids in the colon, especially leucine and valine. Indolic and phenolic compounds produced from the aromatic amino acids by microorganisms are regarded as genotoxic, mutagenic, and carcinogenic substances.

High levels of indole and skatole in the colon of the BCM-fed rat (Fig. 2B) indicated that bacterial fermentation of tryptophan, tyrosine, and phenylalanine was increased.

In the present study, the pyrosequencing analysis confirmed that BCM treatment changed the colonic bacterial composition, even though the abundance of total bacteria was not affected (Fig. 1D). The increase in diversity and the richness index (Table 4) suggest that BCM might affect the community composition of colonic bacteria. Bacteria belonging to the phyla Actinobacteria and Acidobacteria were inhibited by BCM (Fig. 3). Recent studies revealed that the [Fe]- and [FeFe]-hydrogenase gene, which encodes the enzyme involved in hydrogen production, had been found in soil bacteria belonging to the phyla Acidobacteria and Actinobacteria (51, 52). This finding indicated that acidobacteria and actinobacteria might also be potential hydrogen donors in the GIT. The cumulative hydrogen level might cause the feedback inhibition of actinobacteria and acidobacteria.

BCM administration also decreased the abundance of many bacteria belonging to the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria (Fig. 3). Gammaproteobacteria and alphaproteobacteria exhibited slight methanotrophic affinity. It has been shown that they can oxidize at least 0.3% of the methane flux in the artificial rumen fluid (53, 54). Thus, it was reasonable that the abundance of methane-oxidizing proteobacteria would decrease in the BCM group. The abundance of Acetivibrio in the BCM group was significantly increased compared to that in the control group. Studies have found that most members of the Acetivibrio genus are anaerobic carbohydrate-fermenting bacteria, including A. cellulolyticus and A. cellulosolvens (55–57). The increase in Acetivibrio might be involved in the decrease in glucose concentration and carbohydrate metabolism (Fig. 3). Bacteria belonging to the Actinobacteria were significantly decreased in the BCM group. Research found out that bacteria within the Actinobacteria, such as those from the genera Streptomyces and Propionibacterium, can synthesize covalamin-dependent enzymes, including methylmalonyl-CoA mutase and glutamate mutase (58), which were essential for the methionine biosynthesis. BCM can react with reduced covalamin, thereby decreasing methionine synthesis. Since methionine is critical to bacterial DNA synthesis, BCM might decrease the bacterial abundance through the inhibition of methionine synthesis.

Network analysis of taxon cooccurrence patterns has been demonstrated to provide further insight into the structure of complex microbial communities (30). The current study revealed the obvious interdependence of the colonic bacteria in rats by BCM. After BCM administration, the correlation module emerged that Desulfovibrio intestinalis (Deltaproteobacteria), Blautia faecis, and Dorea formigenerans (Clostridia), Pseudomonas geniculata and Pseudomonas putida (Gammaproteobacteria), Streptococcus thermophilus and Lactobacillus sp. (Bacilli), and the Actinobacteria were closely correlated in rats in the BCM group (Fig. 4; see also Table S2 in the supplemental material). B. faecis and D. formigenerans have been reported as potential hydrogen producers (59, 60), while D. intestinalis has been reported as a hydrogen consumer. Other bacteria, like P. geniculata, can also produce hydrogen sulfide (61), while P. putida is effective in hydrogen sulfide removal (62). There were several members of the Actinobacteria, including Bifidobacterium animalis, Collinsella intestinalis, and Olsenella profusa, involved in this modularity class. Although it is not known whether these enteral actinobacterial species produced or utilized hydrogen, the strong correlation between the hydrogen-consuming SRB and hydrogen-producing B. faecis and D. formigenerans suggested that BCM may influence the metabolism in the large intestine through hydrogen utilization.

BCM supplementation did not affect the feed intake and body weight of the rats but significantly altered the gut microbiota and gut metabolism. Colonic bacteria, such as actinobacteria, acidobacteria, and many proteobacteria, were decreased, but the levels of SRB were increased after BCM supplementation. Intestinal metabolism was disturbed by BCM, as evidenced by the decrease in amino acids, BCFA, and carbohydrates in the colon. Amino acid absorption and fermentation were enhanced, which increased potentially detrimental compounds. These findings may provide further information for the use of BCM in animal production.

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