Research Article

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Effect of high-fat diet and growth stage on the diversity and composition of intestinal microbiota in healthy bovine livestock

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Abstract

BACKGROUND: This study aimed to investigate the composition of bacteria in the bovine rectum and their functions during growth, in relation to different diets. Fecal samples were collected from 6-, 12-, 18- and 24-month cattle fed high-fat diet, and healthy female parents fed regular diet. Total DNA was amplified (V3–V4 of 16S rRNA) and submitted to barcode-DNA pyrosequencing. Intestinal microbiota profiles and functions were then analyzed.

RESULTS: A total of 114 512 operational taxonomic units were detected from the 1 802 243 sequences obtained. In 6-month-old and female parent groups, the top three abundant phyla were Bacteroidetes (37.6%, 32.2%), Firmicutes (34.4%, 48.2%) and Proteobacteria (9.1%, 6.3%); in the 12-, 18- and 24-month groups, they were Proteobacteria (45.5%, 47.1%, 38.8%), Firmicutes (27.4%, 22.2%, 20.1%) and Bacteroidetes (14.9%, 19.4%, 17.7%), respectively. *Paludibacter* and *Desulfopila* in abundance showed negative ($P < 0.001$) and positive ($P < 0.05$) correlation, respectively, to cattle weight gain through metagenomic functional prediction of methane, cysteine and methionine metabolism. Meanwhile, cofactor/vitamin and amino acid metabolic processes were significantly higher in bacteria from the regular diet group than high-fat diet groups, with markedly lower cellular processes and signaling, and reduced glycan biosynthesis and metabolism ($P < 0.01$).

CONCLUSIONS: The 6-month cattle and female parents shared similar intestinal bacteria; the community structure of fecal microbiota was significantly affected by high-fat diet in older cattle.

Supporting information may be found in the online version of this article.

Keywords: healthy cattle; intestinal microbiome; growth stages; high-fat diet; metagenomics

INTRODUCTION

A properly functional intestinal tract and optimal microbiota are critical for cattle health, and the host diet has a major influence on gut microbial structure. It was reported that bacteria colonizing in the intestinal tract of cattle profoundly impact animal physiology, nutrition and health,[1–3] as well as human food safety (interacting with pathogens,[4] carrying opportunistic pathogens[5]). Livestock feed formulae are changed and improved continuously for animal health and growth.[6] The fat content of commercial cattle is typically 2–5%, and impacts body performance, weight gain and cold tolerance.[7] The feed additive supplied in fodder positively affected feed digestion and nutrient absorption, presumably changing the composition of cattle intestinal microbiota.[8,9]

Recently, the intestinal microbiota attracts increasing attention from scientists. There are up to 1000 different species and approximately 1012 bacteria per gram of human feces.[10] In cattle, the alimentary tract comprises two biological fermentation pools: the rumen and intestine; the intestinal microbiota interacts with microorganisms in the rumen. The ruminal microbiome has been comprehensively assessed;[6,11] 40% bovine fecal microbiota is characterized by culture-based methods. However, many fecal bacteria are not culturable and are therefore poorly characterized.[12,13] Compared with omnivores and predators, cattle have a richer diversity of fecal microorganisms as herbivores.[14]

The 16S rRNA gene sequencing to assess microbial communities, coupled with the PICRUSt (Phylogenetic Investigation of

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Correction added on 21 June 2017, after first online publication: the "Correspondence to" section contained errors. The errors has been corrected in this version of the article.

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Communities by Reconstruction of Unobserved States) functional inference is considered a more economic and accurate metagenomics approach compared with shotgun metagenomics.[15–17] The predicted functional precision based on 16S rRNA in soil and intestinal microbiota approximates 95%.[18] The aim of this study was to employ barcode pyrosequencing and bioinformatics to comprehensively characterize intestinal microbiota in cattle at different ages fed distinct diets.

MATERIAL AND METHODS

Cattle and sampling

The study was conducted at a scaled bovine livestock farm with more than 10 000 cattle at hand. The cattle strain assessed was a crossbreed of Japanese cattle and Red Angus cattle (male parent) and Qinchuan cows. All cattle were inspected upon receipt to ensure no deformity or early disease signs. Only the animals considered to be healthy were used.

Fecal samples were collected from four age groups, including 6, 12, 18 and 24 months, fed high-fat diet. In each group, five animals were enrolled randomly. Mean weights are shown in Table 1. Another five samples were obtained from female parents at 24 months of age, fed regular diet only. Before 6 months cattle were fed a 100% regular diet and, as age increased, a high-fat diet was proportionally mixed with regular diet – the high-fat diet could reach 80% at 24–28 months (Table 1). The standard operating procedure (SOP) of scaled bovine livestock cultivation was followed throughout the experiment. Strict sterile operation was practiced in collecting specimens in terminal bowel. All fecal samples were placed on ice, transported to the laboratory within 5 h and stored at −80 °C for further use.

Table 1. General condition of cattle in scaled bovine livestock cultivation

Age	Mean weight (kg)	Feed proportion (High-fat[a]/regular[b])
Female parent cattle	700 ± 50	0/100%
before 6 months	300 ± 10	0/100%
6–12 months	360 ± 60	40%/60%
12–18 mouths	560 ± 70	50%/50%
18–24 months	700 ± 50	70%/30%
24–28 months	770 ± 20	80%/20%

[a] High-fat diet contains 85.5% (w/w) corn, 0.5% (w/w) salt, 10% (w/w) sesame dregs and 4% (w/w) pre-mixed roughage, which corresponds to 4.3% total fat, 64.5% total carbohydrate and 11.4% total protein.
[b] Regular diet contains 2.5% (w/w) concentrate, 5% (w/w) ensiling grass, 3% (w/w) vinasse and 5% (w/w) wheat straw, which corresponds to 12–18% crude protein and 82–88% other digestible nutrients.

Stool DNA extraction

Fecal samples were thawed on ice, and stool microbial genomic DNA was extracted using QIAamp DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Total genomic DNA extracts were treated with DNase-free RNase (100 mg L^{-1}) and DNA concentration was determined on a Nanophotometer™ (IMPLEN, Germany). Finally, the extracted DNA was stored at −40 °C until use.

16S rRNA V3–V4 region amplification and sequencing

The V3–V4 region of bacterial 16S rRNA was amplified from the isolated microbial genomic DNA using the following primers: F515, 5′-GTG CCA GCM GCC GCG GTA A-3′; R806, 5′-GGA CTA CVS GGG TAT CTA AT-3′. Polymerase chain reaction (PCR) amplification was carried out in 50 μL reaction mixtures containing 0.5 U ExTag Hotstart DNA polymerase (TaKaRa Inc., Dalian, China), dNTP 50 μmol L^{-1}, 25 μmol L^{-1} of each primer, 5 μL premix buffer (containing 20 mmol L^{-1} $MgCl_2$) and 50 ng DNA. PCR was performed on an automated thermocycler (Bio-Rad MyCycler, USA) for 15 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C; a final extension was carried out for 8 min at 72 °C. Amplicons were visualized on 1.5% agarose and checked for length; products with the desired size (approximately 290 bp) were purified using a QIAquick gel extraction kit (QIAGEN, Germany). DNA quality and amounts were assessed using an Invitrogen Qubit® dsDNA BR kit. The experiments were performed in triplicate. The purified fragments in each sample were normalized and pooled. Pyrosequencing was carried out at the Bioinformatics Research Center of Macrogen (Shenzhen, China) using the Illumina HiSeq 2500 system.[19]

Bioinformatics analysis

Raw Illumina reads were normalized by removing poorly aligned entities; the normalized reads were clustered and aligned. Taxonomic groups were divided by the operational taxonomic unit-based method with similarity not lower than 97%. All OTU sequences with more than 500 reads were selected as input elements in the neighbor-joining model. A phylogenetic tree was generated by the MEGA V6.0 software (www.megasoftware.net). PICRUSt was used to predict the gut microbiota profile. Sequencing data were uploaded to the online galaxy terminal (http://huttenhower.harvard.edu/galaxy/) and subsequently exported to STAMP for further analysis.

Statistical analysis

Discriminant analysis and a multivariable model were performed using JMP pro (SAS Institute Inc., NC, US), STAMP[10] and SPSS V20.0 (IBM Inc., IL, USA) to assess microbial changes from 6 to 24 months as well as bacterial genus prevalence.

RESULTS

Topographical differences in 16S rRNA sequence richness

A total of 1 802 243 sequences with a median length of 290 bp (V3–V4) were obtained and classified as bacteria from 25 fecal samples, among which 1 730 858 (96.0%) were obtained after filtering (Table 2). The rarefaction curves generated by MOTHUR, presenting the numbers of reads and operational taxonomic units (OTUs), suggested a high sampling coverage (99%) (Fig. S1, supporting information). The richness of species was evaluated by ACE, and Chao 1, Shannon and Inverse Simpson indices are shown in Table 2.

Bacterial taxonomic composition and relative abundance during growth

The composition of intestinal microbiota was analyzed based on different taxonomic classifications, at the phylum, class, order, family and genus levels. A total of 40 phyla were found in all samples (Fig. 1A). In the 6-month group, the top three abundant phyla were Bacteroidetes (37.6%), Firmicutes (34.4%) and Proteobacteria (9.1%); older animals (12-, 18-, 24-month groups) showed no differences among them ($P > 0.05$), with Proteobacteria (45.5%, 47.1%, 38.8%), Firmicutes (27.4%, 22.2%, 20.1%) and Bacteroidetes

Table 2. Diversity statistics and sequence quality of samples ($n = 25$)

Groups	Read sequence count	No. of OTUs[a]	Chao 1	Shannon index	ACE
6 months	137 470 ± 21 840	8 710 ± 1 586	12 762 ± 1 975	8.99 ± 0.20	14 199 ± 2 300
12 months	49 199 ± 9 372	5 437 ± 871	10 677 ± 1 567	7.32 ± 1.08	11 480 ± 1 760
18 mouths	50 470 ± 4 123	4 998 ± 593	9 782 ± 1 087	6.97 ± 1.16	10 564 ± 1 169
24 months	48 186 ± 11 462	6 563 ± 2 361	10 461 ± 2 043	9.02 ± 1.88	11 210 ± 2 128
Cows	60 846 ± 5 819	6 956 ± 325	13 174 ± 690	9.81 ± 0.26	13 948 ± 878

[a] OTUs, reading from OUT ID taxonomy data library, not representing the species amounts in the sample.

(14.9%, 19.4%, 17.7%) as the major phyla. In the 24-month female parent group, Firmicutes (48.2%), Bacteroidetes (32.2%) and Proteobacteria (6.3%) were the predominant phyla. Moreover, Verrucomicrobia abundance (1.18 ± 0.39%, 1.78 ± 0.61%, 1.55 ± 0.87%, 1.99 ± 1.23%, 1.76 ± 0.34% in 6-, 12-, 18-, 24-month and cow groups, respectively) was similar in all groups as assessed by one-way analysis of variance (ANOVA) ($F = 0.827$, $P = 0.527$), which may indicate that colonization of Verrucomicrobia in cattle was not affected by diet or age.

The sequences were assigned to 101 different classes (Fig. 1B). In the 6 months group, the top three classes were Clostridia (26.4%), Bacteroidia (23.9%) and Gammaproteobacteria (6.4%); the 12-, 18- and 24-month groups showed no differences among them ($P > 0.05$), with Gammaproteobacteria (42.2%, 43.5%, 28.9%), Clostridia (21.0%, 16.2%, 14.9%) and Bacteroidia (9.3%, 13.1%, 12.6%) representing the most abundant classes. In the 24-month female parent group, Clostridia (40.1%), Bacteroidia (19.1%) and Gammaproteobacteria (3.1%) were the most represented. *Bacillus* abundance among the 12-, 18- and 24-month groups showed a significant difference ($F = 4.138$, $P = 0.013$).

At the order level, sequences were assigned to 176 different taxa (Fig. 1C). Enterobacteriales (38.5%, 39.2%, 19.8%) were the most abundant in the 12-, 18- and 24-month groups, followed by Clostridiales (20.5%, 15.9%, 14.6%), Bacteroidales (9.3%, 13.1%, 12.6%), Pseudomonadales (2.5%, 2.5%, 1.4%), Bacillales (1.55%, 1.6%, 0.9%) and Spirochaetales (0.7%, 2.0%, 1.2%), and obviously these groups have identical top six taxa in order rank. In the 6-month cattle group, Clostridiales (25.9%) was the most abundant order, followed by Bacteroidales (23.9%), Enterobacteriales (4.2%), Spirochaetales (3.3%) and Selenomonadales (1.3%).

At the family level, sequences were assigned to 418 taxa (Fig. 1D). There were 10 families with relative abundance exceeding 1%, comprising 49.2% of all sequences. In the 12-, 18-, 24-month groups, Enterobacteriaceae (32.5%), Ruminococcaceae (7.0%), Prevotellaceae (5.0%) and Lachnospiraceae (4.9%) were the most abundant, followed by Moraxellaceae (2.1%), Porphyromonadaceae (1.8%), Spirochaetaceae (1.3%) and Verrucomicrobiaceae (1.1%) on average. Single-factor variance analysis showed that bacterial composition and abundance in the intestinal flora had no significant differences at the family level ($P > 0.05$) among the 12-, 18- and 24-month groups of cattle. In the 6-month cattle group, Ruminococcaceae (11.5%), Bacteroidaceae (5.4%), Lachnospiraceae (4.7%), Enterobacteriaceae (4.2%) and Spirochaetaceae (3.3%) showed predominance, followed by Prevotellaceae (2.9%), Porphyromonadaceae (2.6%) and Rikenellaceae (2.3%).

At the genus level, more than 797 taxa were detected, with 15 genera whose relative abundance exceeded 1%. The top five most abundant bacterial genera in the 12-, 18- and 24-month groups were *Escherichia* (37.4%, 38.3%, 19.0%), *Acinetobacter* (2.4%, 2.4%, 1.3%), *Alistipes* (0.9%, 1.3%, 0.3%), *Treponema* (0.7%, 2.0%, 1.1%) and *Alloprevotella* (0.4%, 1.5%, 1.2%). No significant differences in bacterial composition were found among the three groups ($P > 0.05$; Fig. S2, supporting information). In the 6-month and female parent groups, the most abundant bacterial genera were similar: *Escherichia* (4.0%, 0.8%), *Bacteroides* (5.4%, 1.0%), *Alistipes* (2.3%, 1.5%), *Oscillibacter* (0.7%, 1.1%) and *Treponema* (3.0%, 0.4%).

Effect of different diets on intestinal bacterial composition

To analyze the contribution of diet in the gut microbiome, microbiota diversity was comparatively assessed in the high-fat diet groups (12-, 18- and 24-month-old cattle, $n = 15$) and regular-diet group (female parent cattle, $n = 5$). Since 6-month cattle had just started to be fed high-fat diet, with milk and regular diet before weaning greatly affecting the gut microbiome, this group was excluded from the analysis. The overall percentages of bacterial populations and inter-phylum variations are shown in Figs 1 and 2. In high-fat diet groups (12-, 18- and 24-month groups), the prevalent phyla were Proteobacteria (43.8%), Firmicutes (23.3%) and Bacteroidetes (17.3%). In the regular-diet group, Firmicutes (48.2%), Bacteroidetes (32.2%) and Proteobacteria (6.3%) were the most represented. In the regular-diet group, prevalence rates of Firmicutes and Bacteroidetes were significantly increased ($P < 0.001$), and Proteobacteria significantly decreased ($P < 0.001$) (Fig. 2); In the 24-month groups, the Firmicutes (48.2%) and Bacteroidetes (32.2%) significantly increased ($P < 0.001$) and Proteobacteria (6.3%) significantly decreased ($P < 0.001$) in regular-diet cattle when compared with high-fat-diet cattle (20.1%, 17.7%, 38.8%) (Fig. 1A).

A phylogenetic tree, which structurally represents high-abundance microbiota in terms of genetic distance, was constructed based on 16S rRNA top OTU sequences (>500 reads) in high-fat and regular-diet cattle groups. Interestingly, OTU distribution and succession showed that the majority of genera in high-fat-diet groups belonged to the Proteobacteria, Firmicutes and Bacteroidetes phyla, while in regular-diet cattle they were Firmicutes, Bacteroidetes and Proteobacteria (Fig. 3).

Effect of growth stage on intestinal bacterial composition

Discriminant analysis was used to assess the variation of intestinal microbiota with age. Figure 4 is a three-dimensional diagram, plotted with bacterial genus abundance as covariate and age as categorical variable. The correlations within each group were analyzed at the genus level; 15 genera were selected with relative abundance comprising 80% of the total flora. A quantitative interaction among each genus's internal community with correlation test ($P < 0.05$, $0.888 \leq |r| \leq 0.999$) (Fig. S3, supporting information) was obtained. With cattle growth, intestinal bacterial genera showed a close relationship. Samples from 6-month cattle were located far

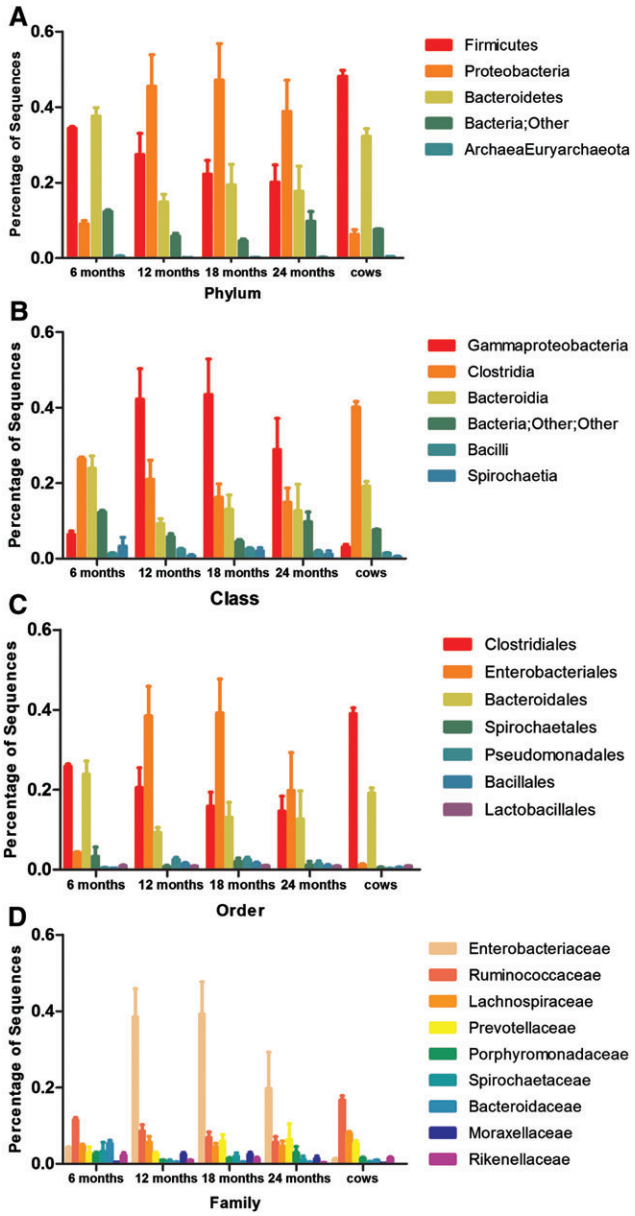

Figure 1. Bacteria phylum (A), class (B), order (C) and family (D) distributions based on 16S rRNA V3–V4 region sequencing of 25 samples from five groups.

from the other groups; 24-month cattle with high-fat diet showed a very similar intestinal microbiota to female parents (24 months) fed regular diet.

The relationship between bacterial species and cattle weight gain

To assess which bacteria contribute most to cattle weight gain, multivariable mixed linear model analysis (weight set as dependent variable) was carried out among 21 genera that were significantly different among groups ($P < 0.05$, ANOVA) (Table 3). After the variable was entered/removed in a stepwise fashion (criteria: probability of F to enter ≤ 0.050; probability of F to remove ≥ 0.100), a predictor ($r = 0.831$) was applied, and the two genera *Paludibacter* and *Desulfopila* were obtained. *Paludibacter* showed a negative correlation with weight ($\beta = -0.693$, $P < 0.001$) and *Desulfopila* a positive one ($\beta = 0.301$, $P = 0.047$). In cattle, H_2S produced by *Desulfopila* in the gut may help digestion of feed proteins;[20] *Paludibacter* could produce methane and waste food energy.[21] Combined with the predicted metagenomics function of methane, cysteine and methionine metabolism (Fig. 5A), this may indicate that increased host body weight was accompanied by reduced amounts of *Paludibacter* and increased *Desulfopila* levels.

Functional structures predicted for bacterial communities by PICRUSt

Compared with the 16S rRNA reference database, two datasets were obtained with the PICRUSt software, which represented the phylum and class levels, respectively. Subsequently, data were exported to STAMP, retaining unclassified reads, and finally obtained KEGG pathway prediction in bacterial communities. With cattle growth, bacterial functions related to the sensory system and cardiovascular disease increased, while immune system and nucleotide metabolism decreased (Fig. S4, supporting information). The KEGG pathways relevant to weight gain were extracted (Fig. 5A); relative abundance of methane production in intestinal microorganisms increased, while amino acid metabolism decreased with cattle growth (Fig. 5B). Finally, we conducted a statistical inference; compared with the regular-diet group, the functions of intestinal bacteria in high-fat diet groups showed significant differences (Fig. 6, Fig. S5, supporting information).

DISCUSSION

Multiple reports have focused on ruminal bacteria, with few assessing cattle intestinal microbiota.[22] Therefore, it is essential to evaluate the composition and function of gut microbiota, to explore its roles in healthy cattle. In this study, fecal samples were collected from cattle at different growth stages and fed distinct diets, and gut microbiota was analyzed. Dominant bacteria phyla, Firmicutes and Bacteroidetes, for instance, in the 6-month group were similar to those in the female parent group, indicating maternal influence on the gut microbiome. The first bacterial encounter occurs during the birthing process, where Firmicutes and Bacteroidetes are the predominant phyla in the vaginal cavity.[23] Significant differences in the fecal flora structure indicated that fecal microbiome diversity after 6 months was changed unpredictably. In the 12-, 18- and 24-month groups, Proteobacteria constituted the most abundant phylum, and ANOVA revealed no differences in the three groups at the family level. This may indicate that maturation of the bovine intestine gradually occurs after 6 months. In 6-month cattle, *Ruminococcus* levels were extremely low (0.03%), while its prevalence in the 12-, 18- and 24-month groups was increased without statistically significant difference (0.20%, $F = 0.945$, $P > 0.05$). Two *Ruminococcus* species found in the rumen were reported to play an important role in isovaleric and isobutyrate acid synthesis for amino acid and lipid production.[24] This may also indicate that stably high colonization of *Ruminococcus* is related to the maturation and development of bovine intestine after 6 months.

The greatest influencing parameters of gut microbial structure with increasing age are environmental factors, especially animal diet. Indeed, dietary intake may alter microbial community diversity, and correlate with health and weight gain. Callaway reported a decreased Firmicutes/Bacteroidetes ratio within the ruminal fluid as dietary supplementation was carried out with distiller's dried grains, with increased proportion of Acinetobacter

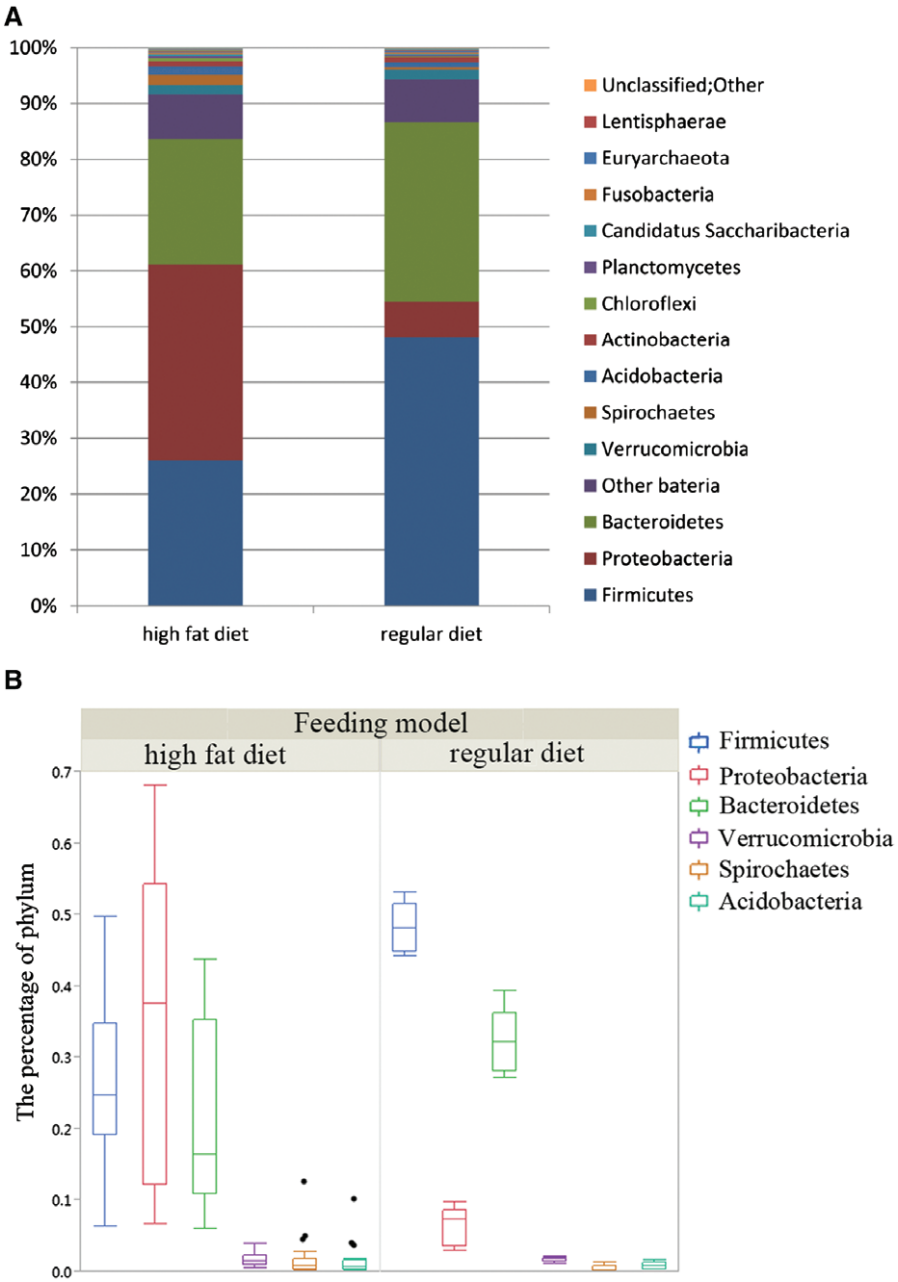

Figure 2. Fecal bacterial populations. Overall percentages of bacterial populations at the phylum level (A) and inter-phylum variation (B) in cattle of high-fat-diet group (12-, 18- and 24-month cattle) and regular-diet group (female parent cows of 24 months).

in feces from animals fed higher-grain diets.[25] Our results showed a ratio of 1.53 in the 12-, 18- and 24-month groups, which was significantly higher than the 0.91 in 6-month cattle ($P < 0.01$), with no difference compared to the value of 1.52 in the female parent group ($P = 0.96$). High Firmicutes/Bacteroidetes ratio was shown to correlate with obesity-associated gut microbiome.[26,27] Fecal samples with obesity microbiota characteristics have a general tendency of growing fat. However, the high Firmicutes to Bacteroidetes ratio in the 12-, 18- and 24-month groups was not accompanied with decreased proportion of Acinetobacter, in disagreement with Callaway's findings; this may be due to the increased proportion of high-fat diet supplementation with age.

It was shown that *Desulfopila* contributes positively, and *Paludibacter* negatively to the host body weight. Sulfur-containing amino acids and sulfate can be metabolized into hydrogen sulfide (H_2S) by *Desulfopila*; the resulting H_2S exerts many effects on the digestive system,[20] including relaxation of the ileal smooth muscle, intestinal secretion stimulation and reduction of colorectal distension-induced visceral pain.[28] In cattle, H_2S produced by *Desulfopila* in the gut may help digest feed proteins, finally increasing the host body weight. *Paludibacter* is an anaerobic bacterium, and related to methane production. In different diets, based on feeding level, fodder composition and digestion, methane would waste 2–15% of food energy.[21] This may explain the negative contribution of *Paludibacter* to weight gain in cattle.

Bacillus subtilis has a therapeutic activity in human intestinal disorders as an anti-diarrhoeal microorganism,[29] and is used as

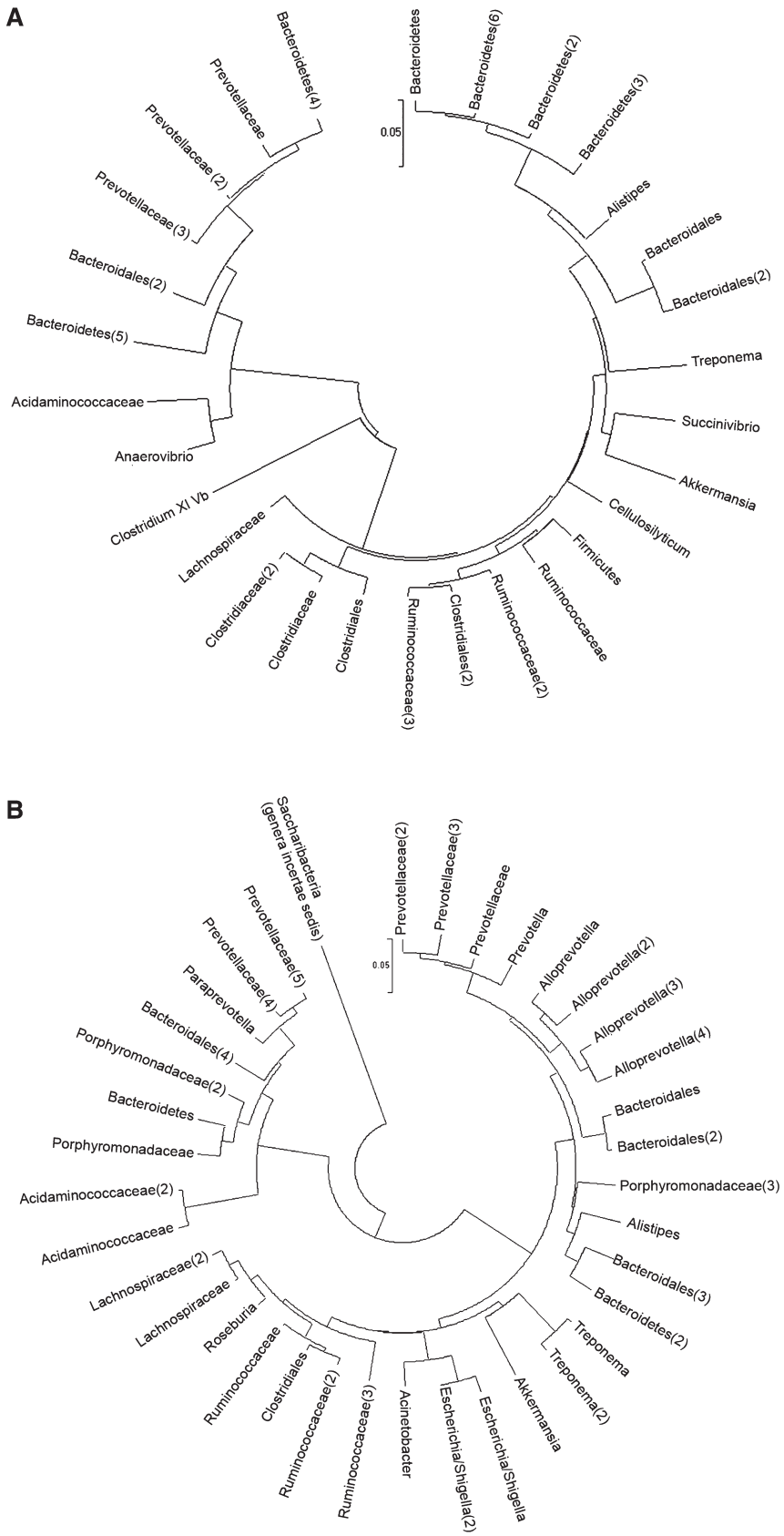

Figure 3. Phylogenetic tree of top read 16S rRNA OTU sequences (greater than 500 reads in total OTUs) of high-fat-diet cattle fecal samples (A) compared with those of the regular-diet fed cattle (B).

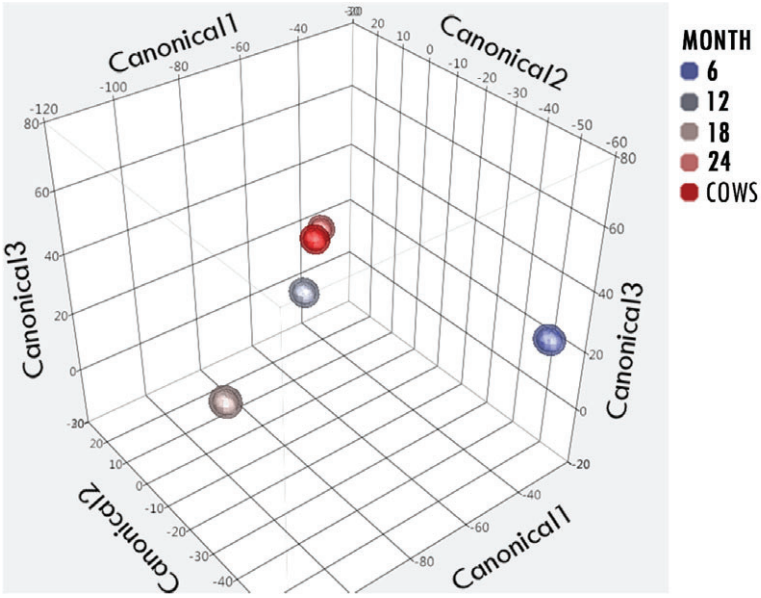

Figure 4. Discriminant analysis was performed in JMP pro (SAS Institute Inc., NC, US) using bacterial genus abundance as covariates and month of life as the categorical variable. The microbial transition of 6-month (blue), 12-month (grey), 18-month (brown), 24-month (orange) and female parents (cows, red) is illustrated by circles.

Table 3. Most abundant genus in the gut microbiota of five groups

	Means (%)				
Genera	6 months group	12 months group	18 months group	24 months group	Cows
Escherichia/Shigella	3.9545	37.4079	38.2841	19.0337	0.7850
Bacteroides	5.3620	0.4283	0.4721	0.4213	0.9565
Acinetobacter	0.4643	2.4378	2.4056	1.3131	0.0659
Alistipes	2.2620	0.9014	1.2539	0.3230	1.5300
Oscillibacter	0.7439	0.3021	0.3024	0.2229	1.1025
Streptococcus	0.3565	0.5992	0.6919	0.5706	0.4455
Akkermansia	0.2047	0.4670	0.5943	0.1499	0.7748
Neisseria	0.0104	0.5905	0.5679	0.4821	0.3598
Spartobacteria	0.0070	0.4166	0.2525	0.6948	0.2601
Paludibacter	1.0432	0.2289	0.0473	0.0107	0.2423
Thioprofundum	0.0937	0.0979	0.1310	1.0942	0.0841
Helicobacter	0.0367	0.3503	0.3538	0.3208	0.2354
Papillibacter	0.1782	0.1526	0.1618	0.0391	0.6027
Ruminococcus	0.0335	0.1918	0.1639	0.2087	0.2451
Leptotrichia	0.0020	0.1716	0.2858	0.1556	0.1188
Victivallis	0.5398	0.0071	0.0024	0.0032	0.1248
Desulfopila	0.0388	0.0516	0.0588	0.4342	0.0396
Pseudoflavonifractor	0.1131	0.0872	0.0415	0.0611	0.3036
Methanobrevibacter	0.0349	0.0732	0.0843	0.1074	0.2726
Rothia	0.0051	0.1355	0.1419	0.1433	0.0992
Granulicatella	0.0004	0.1239	0.1400	0.1299	0.1030

bio-additive in animal feed. In the present study, 15 g per day *Bacillus subtilis* powder containing 10^7 CFU g^{-1} live cells was supplied to cattle from 6 months, and 0.01–0.02% suspected inoculants were detected, which may indicate that *B. subtilis* given orally could colonize in the intestine, but not the predominant bacterial species. Also, the composition of intestinal microbiota was influenced by the diet, age and environment.

As shown above, bacterial taxonomic structure in the 6-month group was dramatically different from those of 12-, 18- and 24-month cattle, and similar to that of the female parent; this may be a post-effect of intestinal microbiome from mother cattle after weaning. Such a notion has been confirmed in humans and pigs.[30,31] However, high-fat feeding was supplied to cattle from 6 months; changes were found in 12-, 18- and 24-month cattle, with no significant differences in intestinal bacterial composition among the three groups. This study aimed to assess microbiota functions of cattle in different growth stages, using PICRUSt predictions for KEGG pathways, which are based on

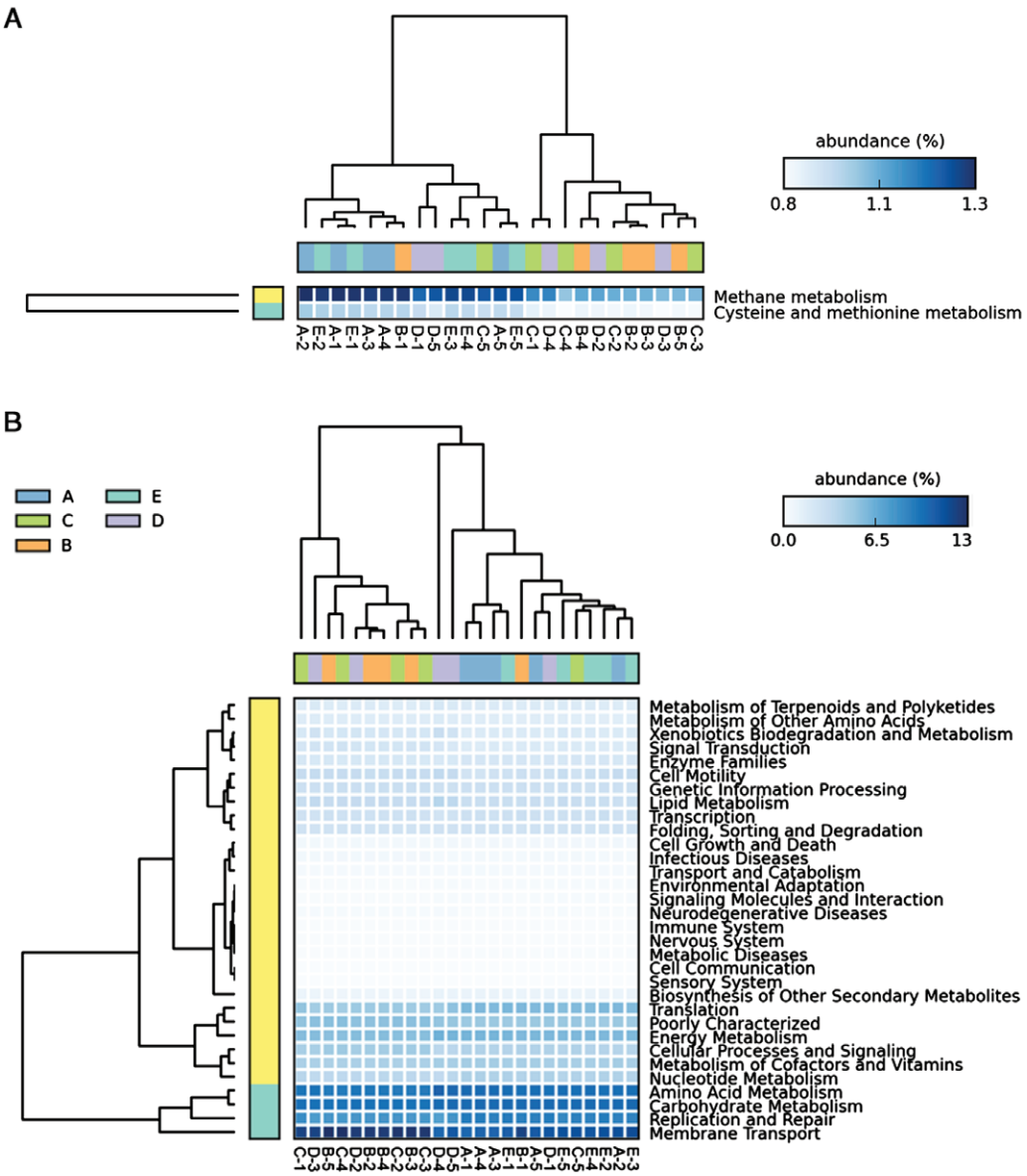

Figure 5. Predicted KEGG pathways correlated with methane production and sulfuretted hydrogen (A), and the full predicted KEGG pathway having significant difference ($P < 0.05$) in intestinal bacteria of various groups at the phylum level (A, 6 months; B, 12 months; C, 18 months; D, 24 months; E, female parent group) (B).

known OTUs assigned to bacterial species (Figure S4, supporting information). Compared with 24-month female parent cattle fed a regular diet, bacterial functions of transcription, metabolism of cofactors and vitamins, amino acid metabolism, and carbohydrate digestion and absorption were much higher. Such an association can be explained by co-occurrence within high-fat diet groups of butyrate-producing bacteria belonging to genera such as *Clostridium*, or Ruminococcaceae members, which were suggested as a candidate taxon associated with butyrate production. The amounts of Ruminococcaceae found in the hindgut, previously reported to be 1.0–2.5% in the rumen,[32] obviously increased with age.

In healthy cattle, maturation of the bovine intestine gradually occurs after 6 months. The high-fat diet could change the proportion of intestinal flora, especially increasing the ratio of Firmicutes/Bacteroidetes, improving the colonization of butyrate-producing *Clostridium* and *Ruminococcus*. Also, *Desulfopila* contributes positively and *Paludibacter* negatively to cattle weight by statistical analysis. The effect of changes in intestinal microbes on physiological function merited further *in vivo* verification. This study provides evidence for associations of microbial ecosystem structure with growth traits in cattle and allows dynamic monitoring of cattle production farms. Further studies, including experimental validation and longitudinal experiments, as well as meta-transcriptomic or metabolomic assessments, are required for a better understanding of the biological relevance of intestinal microbiota composition and functions.

CONCLUSIONS

The composition and diversity of gut microbiota from fecal samples of healthy 6-, 12-, 18- and 24-month cattle fed a high-fat diet were comparatively assessed with a regular-diet group. As shown

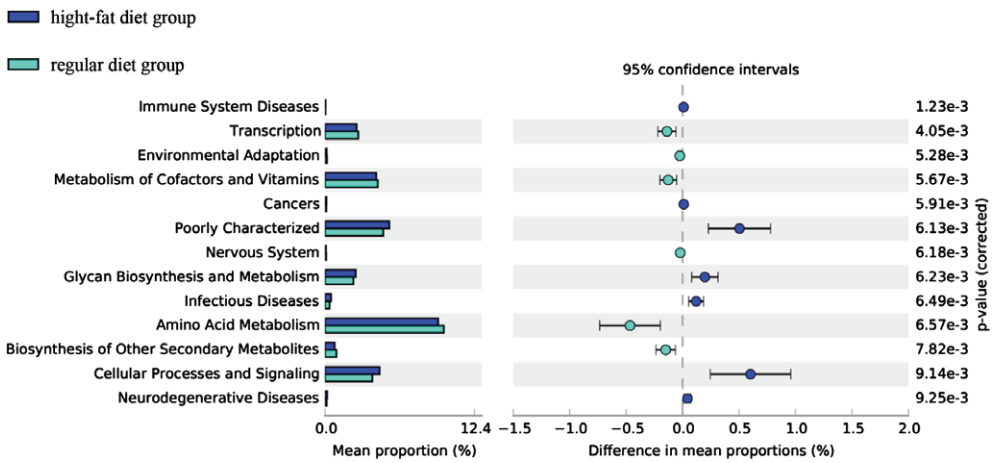

Figure 6. Functional genes of the intestinal microbiome showing significant differences ($P < 0.01$) between high-fat diet and regular-diet groups at the phylum level.

above, the 6-month cattle and female parent groups shared a similar intestinal microbiome; indeed, the intestinal microbiome in heathy cattle tended to be stable after 6 months with abundant Proteobacteria, Firmicutes and Bacteroidetes, as well as cofactor/vitamin metabolism, transport catabolism, and folding and sorting degradation that increased with growth. Diet is a main factor affecting cattle intestinal microbiome. In the high-fat diet group, the proportion of Proteobacteria was significant increased ($P < 0.01$), whereas Firmicutes and Bacteroidetes were markedly decreased ($P < 0.01$). Cofactor/vitamin metabolism and amino acid metabolism of intestinal bacteria were significantly higher, and cellular processes and signaling as well as glycan biosynthesis and metabolism significant lower in cattle fed a regular diet compared with the high-fat diet groups ($P < 0.01$). *Paludibacter* and *Desulfopila* showed negative and positive contributions, respectively, to cattle weight gain. Further biochemical studies and fecal ecological assessments are required to better understand the relationships between microbial communities.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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