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RESEARCH ARTICLE

The Influence of Ketosis on the Rectal Microbiome of Chinese Holstein Cows

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ABSTRACT

This study was conducted to analyze the changes in microbial populations of rectal content of dairy cattle with and without ketosis. The plasma β-hydroxybutyric acid (BHBA) levels of 350 cows were measured and twenty-two post-parturient dairy cows with BHBA levels >1.2 mmol/L were selected and diagnosed with ketosis (KET group). According to statistical pairing rule, 22 dairy cows with BHBA levels <0.60 mmol/l formed the control groups (CON group). The profile of the microbial community of the rectal content samples was detected using high-throughput sequencing analysis of hypervariable V4 region of microbe. The average effective sequences of each sample were 84983, ranging from 64090 to 94470. The Principal Co-ordinates Analysis (PCoA) showed that there were distinctly different clusters of the rectal microbial community between KET and CON cows. Beta diversity analysis was evaluated differences in samples of species complexity. At the phylum level, the percentage of *Euryarchaeota* of the KET cows was less than ($P<0.05$) in the CON group. At the genus level, the percentages of *Ruminococcaceae-UGG-014*, *Methanobrevibacter*, *Erysipelotrichaceae-UGG-009*, and *Atopobium* of the KET cows were less than ($P<0.05$) those found in the CON group. The percentage of *Lachnospiraceae* was greater ($P<0.05$) in KET cows compared with CON cows. *Lachnospiraceae* is related to butyrate production and an increased amount may be an important causative agent of ketosis in dairy cattle. Our findings give a complete picture of current knowledge of the population structure of the rectal microbial ecosystem between KET and CON cows and enhance our understanding about the rectal microbial ecology that may be useful in the prevention of ketosis.

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INTRODUCTION

Ketosis, a metabolic disorder of carbohydrate and fat, is one of the most prevalent and costly metabolic diseases in post-parturient dairy cows, result in high concentrations of ketone bodies in blood, milk and, urine (Zhang *et al*., 2013). There are many microbes in the dairy cow's rumen; in addition, the intestines are also rich in microbes. The gastro-intestinal microbiome is complex and includes many varieties of bacteria, archaea, and fungi that are involved in critical functions within the host, such as immunity, metabolism, digestion (Zhang *et al*., 2015; Rooks *et al*., 2016) and other intestine function (Bogusławskatryk *et al*., 2015) . It has been reported that variations in the microbiota may influence the occurrence of ketosis in dairy cows (Luan *et al*., 2015). Some researchers have used Terminal-restriction fragment length polymorphism (T-RFLP) to investigate the microbe community of ruminant comparisons of small subunit rRNA genes, and correlation between rumen microbe community and marine algae in dairy sheep (Castro-Carrera *et al*., 2014). The conventional method to investigating gastro-intestinal microbe is by isolation and cultivation, which is insufficient. However, the

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composition and diversity of rectal microbe based on high-throughput sequence technology in dairy cows have not been reported. Next-generation sequence of 16S rDNA gene has greatly expanded the ability to obtain more comprehensive and complex information on the microbial community without culture (Logares *et al*., 2014). In this study, we used next-generation sequence of 16S rDNA gene to characterise and compare the microbiota in the rectum of dairy cows with and without ketosis.

MATERIALS AND METHODS

Animal selection and sample collection: Animals in the present study were selected from Jinguang Dairy Cattle Experimental Farm of Guangxi University in China. These Holstein cows were in early lactation (within 60 days postpartum), were fed the same diet, managed in the same routine had the same body condition scores and were producing a similar amount of milk. The β-hydroxybutyric acid (BHBA) levels of blood plasma of 350 post-parturient dairy cows were detected by kit (Randox Laboratories, UK, Cat. No. FA 115) and, the cows with plasma BHBA greater than 1.2mmol/L were diagnosed as ketotic (Y Li, 2016). Twenty-two cows were diagnosed with ketosis, two of which exhibited clinical ketosis (having clinical signs of ketosis and with BHBA 5.3 and 5.6 mmol/L respectively) were merged with the subclinical ketotic cows into one group named KET group. According to the pairing rule such as days in milk (DIM), parity etc., 22 healthy cows were assigned to the control (CON) group. Basic information about the enrolled animals including age, DIM, parity, milk yield (MY) and body condition score (BCS) are shown in Table 1. The rectal content of the KET and CON cows were collected by rectal examination and put into frozen tubes, and stored in liquid nitrogen immediately.

DNA extraction, amplification, and sequencing: Every rectal content sample (200 mg) underwent DNA genome extraction by the SDS method (Natarajan *et al*., 2016). The concentration and purity of DNA samples was monitored on 1% agarose gels. Sterile water was used to dilute DNA to 1ng/μL, and then used a specific primer (515F-806R) with the barcode to amplify the 16S V4 regions, all PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The same volume of 1 x buffer (contained SYB green) was mixed to perform quantification and qualification of the PCR production with PCR products and operated the electrophoresis on 2% agarose gel. Those containing 400-450bp bright main strip samples were chosen for further experiments. The PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany) after being mixed in equidensity ratios. The TruSeq® DNA PCR-Free Sample Preparation Kit was used to generate sequence libraries (Illumina, USA) following the recommendations of manufacturer and index codes were added. The Qubit@2.0 Fluorometer and Agilent Bioanalyzer 2100 system were used to assess library quality, which was sequenced on an IlluminaHiSeq2500 platform to generate 250 bp paired-end reads at last. ALL samples were sequenced by Novogene Bioinformatics Technology Co., Ltd (Beijing, China).

Data analysis: The phylogenetic relationship of different operational taxonomic units (OTUs), and the difference in the dominant species in different groups, and the multiple sequence alignment was conducted by MUSCLE software 3.8.31(http://www.drive5.com/muscle/). Species diversity was evaluated by alpha diversity with QIIME 1.7.0. And the difference between samples of species complexity was evaluated by beta diversity analysis (weighted and unweighted unifrac) with QIIME software 1.7.0.

RESULTS

The diversity of rectal microflora of dairy cows: The 44 rectal content samples were confirmed to be qualified after electrophoresis analysis. The mean effective sequences in each sample were 84983, (range 64090 to 94470). According to the sequences in ≥97% similarity, an average of 1721 OTUs was identified with each sample, ranging from 1276 to 1842 (Fig. 1).

In total, the sequences of rectal content samples of CON and KET group were classified into 27 phyla and 368 genera. The complexity of the microbe in the two groups was evaluated based on alpha-diversity as shown in Fig. 2. Chao 1 (Fig. 2A) and Shannon (Fig. 2B) indexes were used to estimate the community richness and diversity, respectively. The results showed an abundance and diverse range of microbes in both CON and KET groups, however, there was no significant difference.

Comparison of rectal microbial community in dairy cows: In the rectal microbial community, the most abundant taxa microbial at phylum and genus were shown in Fig. 3. The most abundant phyla were *Firmicutes and Bacteroidetes*, accounting for more than 85% of the total microbial sequences in both CON and KET groups. The remaining taxa at phylum were *Proteobacteria*, *Spirochaetes*, *Tenericutes*, *Actinobacteria*, *Euryarchaeota*, *Verrucomicrobia*, *Saccharibacteria* and *Chloroflexi* (Fig. 3A). Both CON and KET groups had similar kinds of microbial phylum, while their proportion of each group differed. There was a difference between CON and KET group at the genus level. *Buchnera* and *Streptococcus* were within the top 10 taxa only in the KET group (Fig. 3B). The remainder taxa were *Ruminococcaceae_UCG-005, Rikenellaceae_RC9_gut_ group, Prevotellaceae_UCG-003, Bacteroides, Christens enellaceae_R-7_group, Treponema_2, Eubacterium coprostanoligenes_group*, *Lachnoclostridium* (Fig. 3B).

The unique and shared OTUs between CON and KET groups were displayed in a Venn diagram to enable comparison (Fig. 4). A total of 2770 and 2663 OTUs were obtained on KET cows and CON cows, respectively. Ketotic and healthy cows shared 2571 OTUs, and the unique OTUs of KET cows and CON cows were 199 and 92, respectively.

A principal component analysis (PCoA) was used to compare the similarities between the microbial community compositions of the 44 experiment animals. The scatter plot based on PCoA scores showed a clear separation of the community composition between the ketotic and healthy cows (Fig. 5). Samples of the KET formed a cluster and distinctly separated from the CON. The result indicated that the microbial composition of the KET was different from that of the CON.

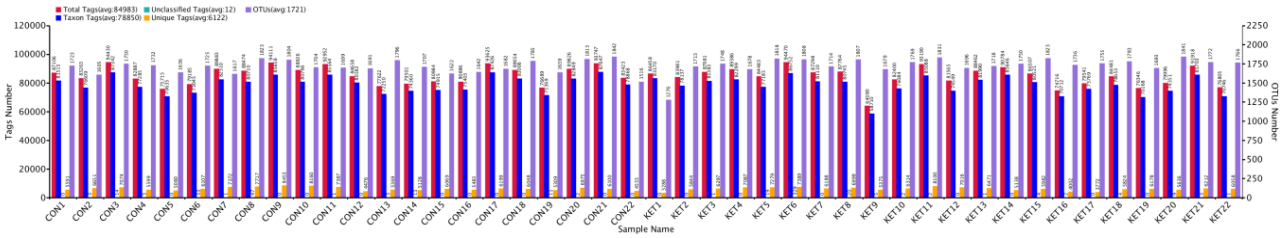


Fig. 1: Complexity of rectal microflora of dairy cows. The results of OTU clustering and annotation of each sample were summarized in this figure.

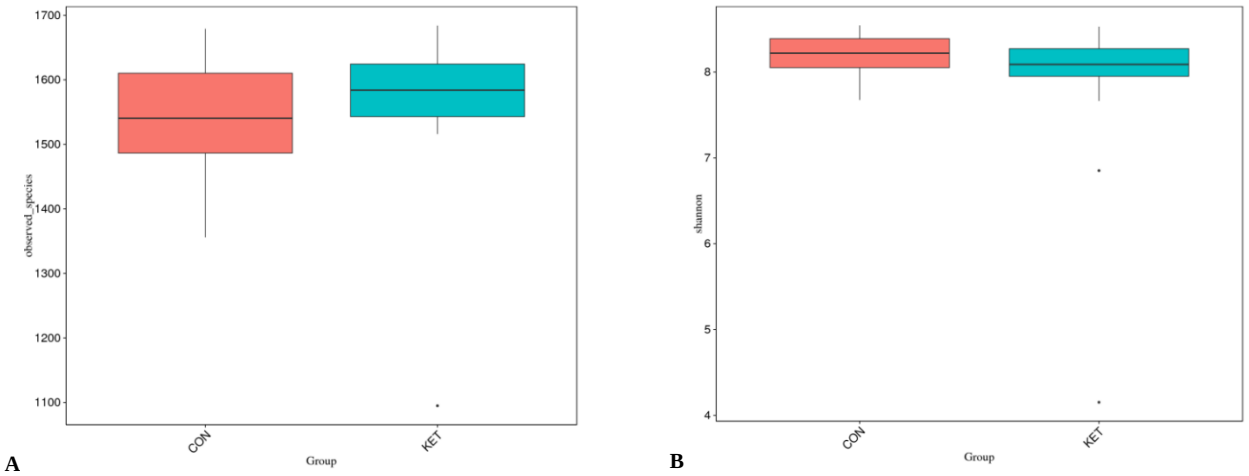


Fig. 2: Index of alpha-diversity. (A)Chao 1 was used to estimated community richness, the complexity of the microbiome in the two groups was estimated at the basis of alpha-diversity. (B) Shannon index was used to estimated community diversity.

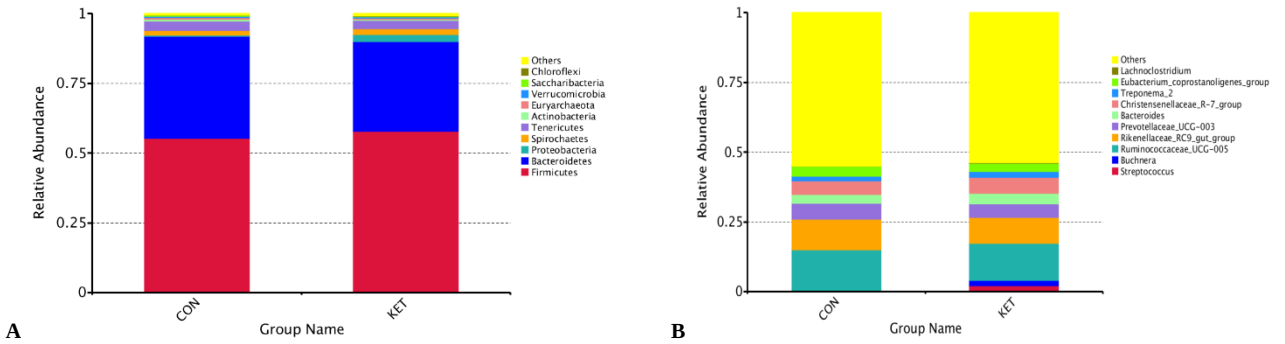


Fig. 3: Composition of rectum microbiota in dairy cows of the CON and KET groups. Each bar represents average relative abundance of each taxa (top 10) within a group. (A) The top 10 microbial taxa at phylum level; (B) The top 10 microbial taxa at genus level.

Table 1: Characteristics of the enrolled animals (Mean±SD)

Group	Age (year)	DIM (day)	Milk yield (kg/d)	BCS	BHBA (mmol/L)	Parity
KET(n=22)	4.9±1.0	23.6±13.3	24.5±2.7**	2.5±0.36	1.9±1.2**	2.9±1.1
CON(n=22)	4.6±0.9	26.5±10.0	28.0±4.3	2.7±0.42	0.5±0.1	2.6±0.9

One-way ANOVA; **P<0.01. Results are expressed as mean±SD.

To identify the specific microbial taxa associated with ketosis, we compared the rectal microbiota of CON and KET cows using the linear discriminant analysis (LDA) effect size (LEfSe) method. A cladogram representative of the structure of rectal microbiota and the predominant microbe was shown in Fig. 6A and 6B; the most significant differences in taxa between the two groups were displayed. The relative abundance of the *Firmicutes* and *Proteobacteria* phylum was significantly higher (LDA>4) in the KET group compared with that of the CON group. As shown in Fig. 6C to K, *Bacilli*, *Lactobacillales*, *Streptococcaceae and Streptococcus* belonged to *Firmicutes* phylum that was significantly higher in KET group than those in CON group; *Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriales*, *Enterobacteriaceae* and *Buchnera* were higher (P<0.05) in the KET group compared with those in the CON group.

In order to identify the significantly different taxa at the different classification level, T-test was performed. As shown in Fig. 7, the different taxa between the CON and KET groups were displayed at phylum and genus level. The proportion of *Euryarchaeota* of the KET group was significantly different from that of the CON group at phylum level (P<0.05) (Fig. 7A). As shown in Fig. 7B, at the genus level, the proportions of *Ruminococcaceae-UGG-014*, *Methanobrevibacter*, *Erysipelotrichaceae-UGG-009*, *Lachnospiraceae-UGG-010*, and *Atopobium* were different between the CON and KET (P<0.05). *Ruminococcaceae_UCG-014*, *Erysipelotrichaceae_UCG-009*, *Lachnospiraceae-UGG-010* belonged to *Firmicutes phylum*, *Methanobrevibacter* belonged to *Euryarchaeota*, and *Atopobium* belonged to *Actinobacteria.*

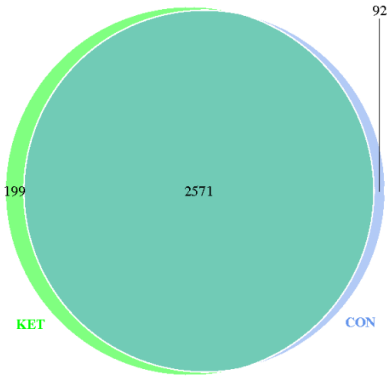


Fig. 4: Unique and shared OTUs in rectal content samples of KET and CON group. The Venn diagram is used to compare with the rectum microbe between CON and KET group.

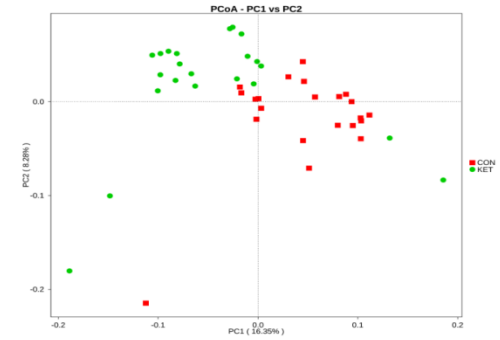
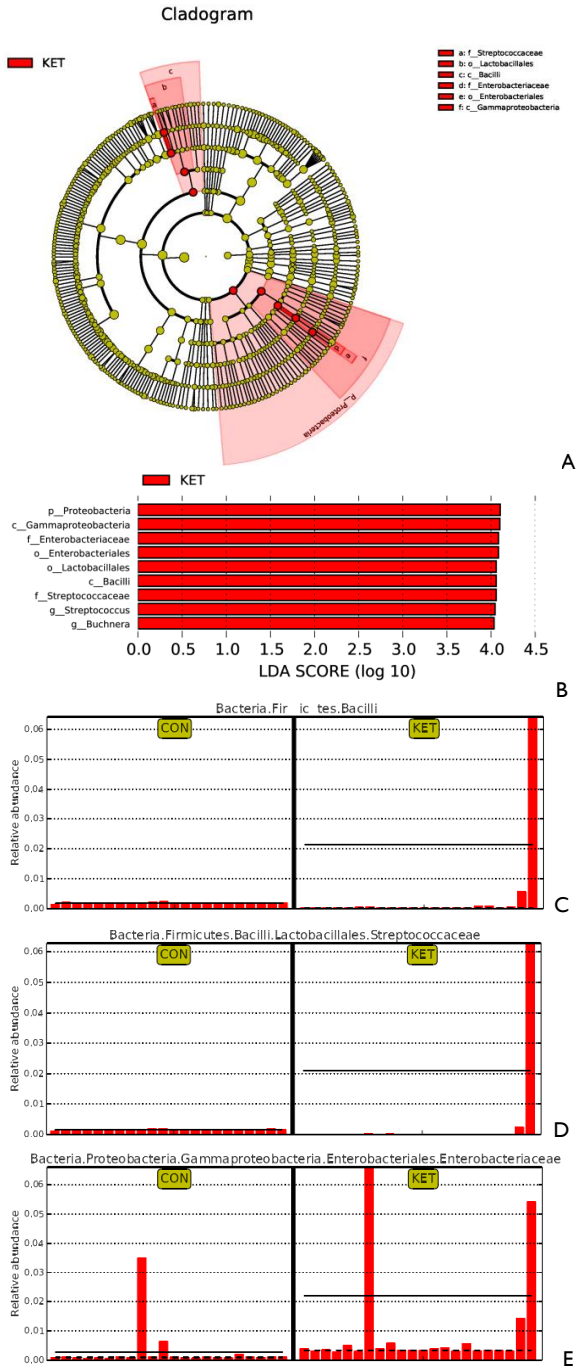


Fig. 5: Principal coordinates analysis (PCoA) of microbial community compositions of dairy cows rectum content based on unweighted UniFrac distance matrix. The scatter plot based on PCoA scores showed a clear separation of the community composition between the ketotic cows and healthy cows.



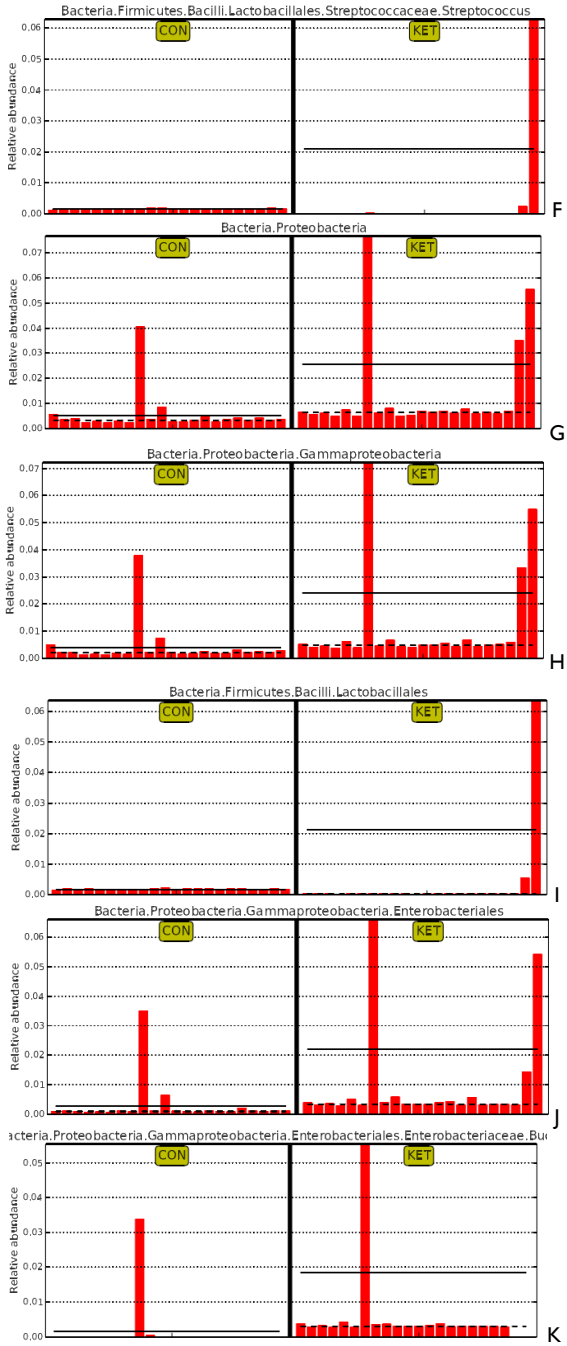


Fig. 6: The difference of microbial taxa abundance of the KET compares to the CON was identified by LEfSe. (A) Taxonomic cladogram obtained from LEfSe sequence analysis. Biomarkers taxa are highlighted by colored circles and shaded areas. (B) The difference in taxa abundance between the KET and CON. The cutoff value of ≧4.0 was used for the linear discriminant analysis (LDA). (C) to (K) Details of the difference in taxa abundance between the KET and CON.

DISCUSSION

Microbial composition in the hindgut region of healthy dairy cows: The rumen is the main place that microbes live in dairy cows and these microbes play an essential role to the health and productivity of the host, such as the degradation and fermentation of cellulose and other polysaccharides (Scharen *et al*., 2017). Gut microbes also underpin the metabolic capability of the host and provide many advantageous effects (Zhang *et al*., 2015). Lots of studies reported that gut harbored a number of microbes that are very important to the host (Marchesi *et al*., 2016; Rooks *et al*., 2016; Sánchez *et al*., 2017). In the present study, the average effective sequences of each sample were 84983, and the microbes from 22 phyla were identified in rectal content samples of healthy cows. The abundant taxa at phylum level (the top 10) were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, *Actinobacteria*, *Euryarchaeota*, *Verrucomicrobia*, *Saccharibacteria*, and *Chloroflexi*, while *Firmicutes* and *Bacteroidetes* made up the vast majority (Fig. 3A). *Firmicutes* and *Bacteroidetes* are also dominant in other animals' gut including humans (Donaldson *et al*., 2016) and fish (Liu *et al*., 2016). *Firmicutes*and and *Bacteroidetes* are the main microbe in most mammals (O Donnell *et al*., 2017), including adult human (Dinan *et al*., 2015). At the genus level, *Ruminococcaceae_UCG-005*, *Rikenellaceae_RC9_gut_ group*, *Prevotellaceae_UCG-003*, *Bacteroides*, *Christens enellaceae_R-7_group*, *Treponema_2*, *Eubacterium_ coprostanoligenes_group*, and *Lachnoclostridium* (Fig. 3B) were dominant in dairy cows' rectal contents. *Ruminococcaceae* could break down cellulose and disaccharide to formic acid and acetic acid (Gagen *et al*., 2015). The diet of dairy cows is rich in cellulose and carbohydrates which are digested by ruminal microbes.

Comparison of rectal microbes between the KET and CON groups: It is generally accepted that the vertebrate gut microbiome plays a vital role in host disease (Gao *et al*., 2017), which is now attracting increased attention regarding its role in the diseased animal. Ketosis is a common metabolic disease of the post-parturient in dairy cow which leads to reduced milk yield, reproductive performance and, increased occurrence of other diseases (Raboisson *et al*., 2014). It is recognized that ketosis occurs because of excessive mobilization of fat which may induce an imbalance in hepatic carbohydrate and fat metabolism, characterized by elevated concentrations of ketone bodies (BHBA, acetoacetate, and acetone) and non-esterified fatty acids (Zhang *et al*., 2016). Luan *et al.* (2015) reported that cows fed on DFM (direct-fed microbials contained Bacillus pumilus 8G-134 tended to have higher feed conversion and to have a lower prevalence of subclinical ketosis. These studies suggested that microbes play a very significant role in the occurrence of ketosis. As shown in Fig. 5, a scatter plot of KET and CON samples based on PCoA scores shows a clear partition of the community composition between the ketotic cows and controls. It indicated that there was a significant difference between the microbial community composition of the KET and the CON cows. Further more, the results of the T-test at the phylum level (Fig. 7A) showed that the proportion of *Euryarchaeota* in rectal faeces of the KET cows was significantly decreased compared to the CON cows. *Euryarchaeota* is implicated in the production of methane through the reduction of CO_2 and H_2 (or formate) (Gaci *et al*., 2014). It has been reported that increasing the forage in the diet of *Bubalusbubal* is could increase the proportion of *Euryarchaeota* in the rumen because the digestion of forage produces CO_2 and H_2 (Singh *et al*., 2015). In the present study, the percentage of *Euryarchaeota* in rectal content of ketotic cows was decreased which may indicate a lack of CO_2 and H_2 from forage digestion, which is likely due to the reduced dry matter intake of ketotic cows. In addition, the percentage of *Methanobrevibacter*, which is a methanogenic microbe, also reduced in dairy cows with ketosis (Fig. 7B), the reason for which may be the same as for the *Euryarchaeota*. The proportion of *Ruminococcaceae* of the KET cows was also different from the CON cows based on the T-test (Fig. 7B). *Ruminococcaceae* is dominant in the rectum of dairy cows (Fig. 3A). It is also dominant in pigs (Argüello *et al*., 2018), and plays a protective role in cirrhotic human patients (Argüello *et al*., 2018). The abundance of *Ruminococcaceae* remarkably reduced in chronic heart failure human patients compared to the controls at the genus level (Luedde *et al*., 2017). The same occurrence is seen in the abundance of *Ruminococcaceae* in dairy cows with ketosis (Fig 7B). This may indicatethat *Ruminococcaceae* may play an essential role in maintaining a healthy host. The proportion of *Erysipelotrichaceae-UGG-00*9 of the KET cows was significantly decreased (Fig. 7B). It has been reported that abundant *Erysipelotrichaceae* of gut microbiota was related to metabolism syndrome and impaired versus normal glucose metabolism in older adults (Lippert *et al*., 2017). Long-chained fatty acids may cause an increased abundance of *Erysipelotrichaceae* during the establishment of the human gut microbiota as has been demonstrated in germ-free mice (Nejrup *et al*., 2017). *Erysipelotrichaceae* may metabolise fatty acids, and ketosis may be associated with a decreased amount of *Erysipelotrichaceae* in the gut. Some *Lachnospiraceaes can* produce butyrate by the butyrate kinase pathway or the butyryl-CoA or acetate CoA transferase pathway (Flint *et al*., 2015) and ketosis occurred with the increase of blood BHBA level. The result based on T-test displayed a significant increase in the abundance of *Lachnospiraceae* in the KET compared with the CON groups. It is proposed that *Lachnospiraceae* may be the main microbial taxa that correlates ketosis. To our knowledge, this is the first comprehensive study with high-throughput analysis of rectal microbiota in dairy cows with and without ketosis. These results indicate a strong influence of the ketosis on the diversity and structure of rectal microbiota.

Conclusions: In the current study, the structure of the rectal microbiota of the ketotic cows was different from that of healthy cows. At phylum level, the percentage of *Euryarchaeota* of the KET cows was significantly decreased compared with that of the CON cows. At the genus level, the percentages of *Ruminococcaceae-UGG-014*, *Methanobrevibacter*, *Erysipelotrichaceae-UGG-009*,

and *Atopobium* were increased (P<0.05) and the percentage of *Lachnospiraceae* was decreased (P<0.05) in the KET cows compared with that of the CON cows. *Lachnospiraceae* is related to butyrate production, the increased percentage of *Lachnospiraceae* in cow's rectal content may play an essential in ketosis.

Animal care: All the experimental procedures were assessed and approved by the Ethics Committee on Animal Experiments of Guangxi University and the care and use of animals complied with the local law and guidelines on animal experiments (Approval No. GXU2016-006).

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Authors contribution: YH, YL and BH conceived and designed the study. YH, JH, MMA, HY, PW, PZ, YLD, LH, WS and XZ executed the experiment and acquired the data. YFH and YL interpreted and assay the data and drafted the manuscript. BH and MMA critically revised the manuscript.

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