Rumen Microbiota Alterations During Ketosis is Associated with the Development of Mastitis in Dairy Cows

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Research Article

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Abstract

**Background:** Mastitis is the most serious disease endangering animal husbandry, especially dairy farming. Clinical investigations indicated that cows suffering from ketosis have a higher probability of mastitis. Rumen microbiota is closely related to ruminant health. However, it is not clear what role it plays in this process.

**Results:** The microbiota in rumen fluid and milk from ketosis cows were determined by 16S rRNA gene sequencing. The results showed that the richness of bacterial community both in rumen and milk were changed in ketosis cows. The abundance of genus *Prevotella, Ruminococcus, Succinivibrionaceae_UCG-001* and *Streptococcus* in rumen fluid from ketosis cows decreased significantly and were negatively correlated with blood BHBA and milk SCC. In contrast, the abundance of genus *Luteimonas, Thermomonas, Christensenellaceae_R-7_group, Rikenellaceae_RC9_gut_group, NK4A214_group, Paracoccus, Acetitomaculum, Prevotellaceae_UCG-003, Deinococcus, Saccharofermentans* and *Butyrivibrio* in rumen fluid from ketosis cows increased significantly and were positively correlated with blood BHBA and milk SCC. In addition, the abundance of *F082* and *Thermomonas* were increased, while the abundance of genus *Acinetobacter* and *UCG-005* were reduced both in milk and rumen fluid in ketosis cows than healthy cows.

**Conclusions:** Ketosis in dairy cows is capable of inducing mastitis. The rumen microbiota of ketotic cows changed significantly and is associated with the development of mastitis. Targeting rumen microbiota regulation may be a promising strategy to prevent metabolism disorder and its secondary diseases in dairy cows.

**Background**

Mastitis is an inflammatory disease in dairy cows and commonly believed to be triggered by pathogens derived from infectious and environmental bacteria, with high incidence and prevalence[1, 2]. In addition, clinical investigations and researches have shown that the metabolic diseases induced by negative energy balance (NEB) in the early postpartum period of cows, especially ketosis, are commonly thought to be related to mastitis [3, 4].

Ketosis is a very frequent metabolic disorder in dairy cattle during early lactation, resulting in lower milk production and impaired fertility[5, 6]. This disorder is characterized by the accumulation of ketone bodies, mainly β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), acetoacetate, and acetone, distributed in blood, milk and urine[7]. Blood BHBA, the predominant and more stable circulating ketone body, is used to diagnose ketosis in almost any dairy herd[8]. Mounting evidence suggests that high levels of BHBA may be a risk factor in the development of mastitis in ketosis cows. Jánosi et al.[3] found that the risk for clinical mastitis elevated with increased blood BHBA levels. Moyes et al.[9] also found that the clinical mastitis cows had higher blood NEFA and BHBA levels than healthy cows before mastitis, indicating that NEFA and BHBA in plasma may be potential markers for mastitis in early lactation.
Suriyasathaporn et al.[4] suggested that the impairment of the udder defense mechanism in NEB cows seemed related to hyperketonemia, which eventually resulted in the increased incidence rate of mastitis. Subsequently, Grinberg et al.[10] found that BHBA abrogated formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic *Escherichia coli*, which might explain the increased susceptibility of ketotic cows to mastitis and other infectious conditions. Moreover, high concentrations of BHBA after calving may exert a chemoattractant effect that possibly plays an important role in the onset of the inflammatory process in dairy cows[11]. Studies also suggested that both BHBA and NEFA at higher concentrations could induce cow hepatocyte inflammatory injury through the nuclear factor-kappaB (NF-κB) signaling pathway and increased the release of pro-inflammatory factors, which might be activated by oxidative stress[12–14].

In addition, evidence showed that the production of ketone bodies was closely related to the proportion of volatile fatty acids (VFAs) in rumen. An increase of ketone body precursors such as butyrate in the intestine of nonruminants or in the rumen of ruminants enhances ketogenesis[15]. Intraruminal VFAs production mainly comes from the fermentation of rumen microbes on feedstock, especially a large number of carbohydrates, providing up to 75% of total metabolizable energy in ruminants[16]. Accordingly, we speculate that the occurrence of mastitis induced by ketosis may be related to the change of rumen microflora.

The rumen, a pregastric anaerobic chamber, is colonized by a complex and diverse microbial community, 95% of which are from bacterial domain[17]. The ruminal microbiome structure is closely related to production traits such as milk production and composition[18]. Ma et al.[19] found that the transfer of fecal microbiota from mastitis cows to germ-free mice resulted in significant mastitis symptoms and inflammations of many tissues in the latter, suggesting that dysbiosis of intestinal microbiota may be one cause of mastitis. Zhong et al. [1] showed an significant difference in rumen microbiota among cows with different SCC, suggesting that rumen microbes were associated with health status of mammary gland. Some clinical trials have shown that oral probiotics supplementation could effectively control clinical mastitis, suggesting that the mechanism of mastitis protection might be achieved via the host gut microbiota[20]. Our previous studies also indicated that the dysbiosis of intestinal microflora could induce inflammation of the mammary gland[21, 22].

Therefore, we hypothesized that ruminal dysbacteriosis developed in ketosis cows and contributed to the occurrence of mastitis. To test this hypothesis, dairy cows with ketosis in the perinatal period were selected. The16S rRNA gene sequencing was performed to investigate the rumen and milk bacterial composition. The current study provides a better understanding of the association between ketosis and mastitis based on rumen and milk microflora analysis. Understanding the composition of ruminal microbiota during ketosis and mastitis will help to develop strategies to modulate the microbiome and enhance dairy cows health and production in early lactation.

**Results**
Effects of Ketosis on Milk Composition in Dairy Cows.

In the cows with ketosis, blood BHBA concentrations, with an average of 3.03 ± 0.22 mmol/L, were significantly higher, compared with the values in the healthy cows (Fig. 1A). The milk SCC in all of the ketosis cows were higher than 500,000 cells/mL, with an average of 464.3 ± 152.3 cells/mL (Fig. 1B). In addition, milk components analysis showed that except the proportion of milk protein remained unchanged, the percentage of lactose decreased significantly, while milk fat, urea nitrogen, as well as the ratio of milk fat to protein increased significantly in ketosis cows than healthy cows (Fig. 1C-G).

Microbiota 16S rDNA Amplicon Sequencing in Rumen Fluid and Milk in Dairy Cows.

We analyzed 36 samples by 16S rDNA amplicon sequencing, including 10 rumen fluid samples and 10 milk samples from ketosis cows, 8 rumen fluid samples and 8 milk samples from healthy cows. Overall, 8,372 OTUs were identified from the bacterial 16S rDNA profile in rumen fluid. At the phylum and genus level, a total of 72 phyla and 1026 genera were observed. A total of 10,623 OTUs were identified from the bacterial 16S rDNA profile in milk. At the phylum and genus level, a total of 89 phyla and 1193 genera were observed. To confirm adequate sequencing depth for these analyses, we generated the species accumulation boxplot for all of the samples and found our coverage to be sufficient for further analyses (Additional file 1).

Changes of Bacterial Community in Rumen Fluid and Milk between Ketosis and Healthy Cows.

The bacterial richness in rumen fluid and milk was estimated by Chao 1 and ACE index. Combined with Wilcoxon rank sum test, the richness of rumen microflora in ketosis cows was significantly lower than in healthy cows, while the richness of milk microflora in mastitis cows was significantly higher than in healthy cows (Fig. 2A-B). The bacterial diversity in rumen fluid and milk was estimated by Shannon and Simpson index. The result showed that there was no significant difference in rumen fluid and milk between ketosis and healthy cows (Fig. 2C-D). PCoA of the weighted UniFrac metric revealed a shift of community diversity among different groups along principal coordinates PC1 and PC2 (Fig. 3A). NMDS ordination performed on the Bray-Curtis dissimilarity showed that the bacterial community profiles of rumen fluid in ketosis cows was clear dissimilar from healthy cows (Fig. 3B).

Rumen Fluid and Milk Microflora at Phylum and Genus levels between Healthy and Ketosis Cows.
The relative abundance of the main bacterial taxa at different taxonomic levels in rumen fluid between ketosis cows and healthy cows were shown in Additional file 2. Both rumen and milk bacterial community were dominated by the following 5 bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteriota* and *unidentified_Bacteria* (Fig. 4A), accounting for more than 90%. However, the most abundant bacterial taxa in rumen microbiota were *Firmicutes* and *Bacteroidetes*. The ratio of *Firmicutes* to *Bacteroidetes* has traditionally been considered as a biomarker for metabolic potential of the gut microbiota[23]. We found that the *Firmicutes* to *Bacteroidetes* ratio significantly increased in ketosis cows than healthy cows(1.41 in KR vs 0.96 in HR)(Fig. 4B). At the genus level, the predominant genera in rumen fluid were *Prevotella*, *Aeromonas*, *Succinivibrio*, *Luteimonas*, *Thermomonas*, *Christensenellaceae_R-7_group*, *Ruminococcus*, *Rikenellaceae_RC9_gut_group*, *NK4A214_group* and *Kocuria*, while in milk were *Streptococcus*, *Vibrio*, *Shewanella*, *Prevotella*, *Staphylococcus*, *Lactobacillus*, *Coxiella*, *Escherichia-Shigella*, *Acinetobacter* and *Ruminococcus* (Fig. 4C-D). Among the ruminal bacterial community, the single most abundant genus was *Prevotella* and its abundance significantly decreased in ketosis cows. The microbiota of milk samples was dominated by the genus *Streptococcus*.

**Differential Bacteria in Rumen Fluid and Milk between Ketosis and Healthy Cows.**

The distribution of common and unique OTUs among different samples is illustrated by Venn diagrams. In rumen fluid samples, there were 3154 OTUs uniquely present in healthy cows, 899 OTUs uniquely present in ketosis cows and 3564 OTUs shared between the ketosis and healthy cows (Fig. 5A). In milk samples, there were 1549 OTUs uniquely present in healthy cows, 2626 OTUs uniquely present in ketosis cows and 5856 OTUs shared between the ketosis and healthy cows (Fig. 5B). Moreover, Venn diagram showed that a total of 4608 OTUs shared by the rumen and milk samples in healthy cows, while 2957 OTUs shared by the rumen and milk samples in ketosis cows (Fig. 5C-D). MetaStat analysis showed that in the rumen fluid microbiota, at genus level, the relative abundance of *Prevotella*, *Ruminococcus*, *Succinivibrionaceae_UCG-001* and *Streptococcus* were significantly decreased in ketosis cows, while the relative abundance of *Luteimonas*, *Thermomonas*, *Christensenellaceae_R-7_group*, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Paracoccus*, *Acetitomaculum*, *Prevotellaceae_UCG-003*, *Deinococcus*, *Saccharofermentans*, *Butyrivibrio* and other low abundance genus were significantly increased in ketosis cows (Fig. 6A). LEfSe analysis was used to find biomarker in ketosis cows. The results showed that *F082*, *Luteimonas*, *Thermomonas*, *Christensenellaceae_R-7_group*, *Rikenellaceae* and *Lachnospiraceae* were enriched in rumen fluid from ketosis cows, while *Prevotella* was enriched in rumen fluid from healthy cows. (Fig. 6B-C).

Furthermore, LEfSe analysis revealed that *Staphylococcus* and *F082* were enriched in milk from ketosis cows, while *Corynebacterium*, *UCG-005*, *Acinetobacter* and *Coxiella* were enriched in milk from healthy cows(Fig. 7A-B). Interestingly, we found that both *F082*, an unclassified bacterial family assigned to *Bacteroidales*, and *Thermomonas* were enriched in milk and rumen fluid from ketosis cows. In addition, MetaStat analysis revealed that the abundance of *Acinetobacter* and *Oscilllospiraceae_UCG-005* in milk
were reduced simultaneously with those in rumen in ketosis cows. However, in ketosis cows, the abundance of *Corynebacterium* was decreased significantly in milk, while it was increased significantly in rumen (Fig. 7C).

**Correlation Analysis of Rumen Microflora with Blood BHBA and Milk Components.**

Correlation between the blood BHBA and milk components and bacterial taxa with top 35 relative abundance at genus level were illustrated in a heatmap (Fig. 8). The abundance of *Luteimonas*, *Thermomonas*, *Christensenellaceae_R-7_group*, *Kocuria*, *Paracoccus*, *Corynebacterium*, *Acetitomaculum* and *Deinococcus* showed significantly positive correlation with blood BHBA concentration. However, the abundance of *Prevotella*, *Ruminococcus*, *Succinivibrionaceae_UCG-001* and *Streptococcus* correlated negatively with blood BHBA concentration, as did the lower taxa *Erysipelotrichaceae_UCG-002*, *Syntrophococcus*, *Lactobacillus* and *Dialister*. Highly consistent with the MetaStat analysis, we observed a higher abundance of the bacteria positively correlated with blood BHBA in ketosis cows than in healthy cows, while we observed a lower abundance of the bacteria negatively correlated with blood BHBA in ketosis cows than in healthy cows (Fig. 6A). Interestingly, most of these bacterial genera significantly related to blood BHBA are related to the SCC in milk, and their correlation are consistent. Moreover, the milk fat, milk urea nitrogen increased with BHBA at the same time, so did the same correlation with these bacteria. In contrast, *Prevotella*, *Ruminococcus*, *Succinivibrionaceae_UCG-001* and *Streptococcus* showed significantly positive correlations with milk lactose.

**Discussion**

Mastitis is a multietiological and complex disease, which is defined as inflammation of mammary glands[24]. It is recognized that mastitis mainly resulted from infection of pathogenic microorganisms. However, mastitis can be induced by a variety of factors, and many other clinical diseases tend to increase the susceptibility and incidence of mastitis. According to previous studies, the excessive NEB in perinatal cows leads to decreased immunity. Hyperketinemia and high concentrations of circulating NEFA in plasma induced by ketosis lead to oxidative stress and induce liver injury and systemic inflammatory response, subsequently resulting in the elevated prevalence of mastitis[25, 26]. In our study, we found a significant increase of blood BHBA and milk SCC in all of the ketosis cows (Fig. 1A-B). Previous studies indicated that BHBA could affect the chemotaxis and bactericidal function of neutrophils and might be related to the impairment of the udder defense mechanism in ketosis cows[4, 10, 11]. The elevated blood BHBA levels in dairy cows exactly increased the risk for clinical mastitis[3, 9]. Evidence showed that the production of ketone bodies was closely related to the proportion of VFAs in rumen. High acetic acid and butyric acid, low propionic acid fermentation promotes ketoplasia. An increase of ketone body precursors such as butyrate in the intestine of nonruminants or in the rumen enhances ketogenesis[15]. Intraruminal
VFAs mainly come from the fermentation of rumen microbes on carbohydrates in feedstock[16]. The concentration and proportion of different VFAs are significantly related to the composition of the bacterial community in rumen[27]. Therefore, we speculate that the occurrence of mastitis induced by ketosis may be associated to the change of rumen microbial community. In our study, dairy cows with ketosis and mastitis were selected to collect the milk and rumen fluid samples. Through 16S rRNA gene sequencing and multiple analysis on the rumen and milk microbiome, we found a significant difference in rumen microbiota between ketosis and healthy cows. Some bacteria in rumen were closely related to blood BHBA and milk SCC.

The complex rumen microbiome enables the ruminants to digest their plant feed through microbial-mediated fermentation[28]. It is closely related to production traits such as milk production and composition[18]. Moreover, numerous studies have found that the rumen microbial community and its metabolites are involved in the occurrence of inflammation and the permeability of gastrointestinal tract, which may be associated with some diseases in ruminants[29]. In this study, we found that Bacteroidota, Firmicutes and Proteobacteria were the most abundant phyla, accounting for more than 85% of rumen bacterial community in dairy cows. However, the relative abundances of them in ketosis cows changed significantly, which especially characterized by a significant decrease of Bacteroides (Table S1). The Firmicutes to Bacteroidetes ratio has traditionally been considered as a biomarker for metabolic potential of the gut microbiota[23]. The ratio of Firmicutes to Bacteroidetes was found to be strongly correlated with daily milk fat yield[30]. Moreover, the Firmicutes to Bacteroidetes ratio indicates the proportion of different VFAs in rumen such as butyrate produced especially by Firmicutes, propionate by Bacteroidetes[31]. Therefore, in our study, the increase of blood BHBA and milk fat observed in ketosis cows may be related to elevated Firmicutes to Bacteroidetes ratio. The most noticeable shifts in rumen microbiota of ketosis cows were a decrease in the proportion of Prevotella assigned to Bacteroidetes and an increase in Proteobacteria including Luteimonas, Thermomonas and Paracoccus (Table S1). Especially, Prevotella, the most dominant genus in ruminal bacterial community, a multifunctional producer using a large variety of substrates as energy sources [32], was positively correlated with propionate concentration and proportion[33]. Xanthomonadaceae bacteria including Luteimonas and Thermomonas, generally are considered plant pathogens and their abundance are very low in healthy cows. However, bacteria from Luteimonas, Thermomonas and Paracoccus as nitrate reducing bacteria, participating in the absorption and utilization of nitrogen in rumen, likely relate to the increase of milk urea nitrogen in ketosis cows [34, 35]. Similarly, Rikenellaceae_RC9_gut_group may play important roles in utilizing the carbohydrate and nitrogen in ruminants[36]. Among the Firmicutes, Christensenellaceae_R-7_group, Acetitomaculum and Butyrivibrio significantly increased in ketosis cows, whereas Ruminococcus significantly decreased. Lachnospiraceae bacteria including Acetitomaculum and Butyrivibrio, with the high relative abundance in rumen fluid, are considered to have the characteristic of butyrate-producer[37, 38]. Bacteria from the family Christensenellaceae which produce acetic acid and a small amount of butyric acid as fermentation end products likely play an important role in ketogenesis[18]. Studies also found that Christensenellaceae_R-7 related to malnutrition, suggesting that the bacteria may harm the energy absorption and use of the host[39]. Typically, the postpartum high-grain
feed, to meet the energy and protein demands, leads to shifts in bacterial populations with increases in *Bacteroidetes* and reductions in *Firmicutes* [17]. In our study, we observed the opposite phenomenon. Compared with healthy cows, the ruminal microbiota of ketosis cows seemed to fail to adapt to the change in postpartum diet, producing insufficient VFAs, and ultimately leading to NEB [27]. Meanwhile, the richness of microbiota in ketosis cows decreased significantly, while there was no significant change in diversity, suggesting that ketosis cows might be accompanied by a significant decrease in appetite or dry matter intake (DMI). Degradation of diets by rumen microbes can be said to be regimented due to their preference for different feed structures and substrates. Similarly, individual variation of animals plays an important role in deciding the rumen microbiome of ruminants even when fed similar diets [40]. Perhaps it is the reason why some cows are more susceptible to ketosis in a herd. In a new study about predictive ability of rumen microbiome for subclinical ketosis, researchers found that rumen microbial composition explains a larger proportion of the variation in milk concentrations of acetone and BHBA than do host genetics. *Prevotellaceae* and *Ruminococcaceae* were found to be highly significantly associated with BHBA and acetone in milk. With increasing levels of BHBA and acetone, all of the top bacterial OTUs were depleted in relative abundance, whereas the archaeal OTUs showed enrichment in relative abundance [41]. It is largely consistent with our findings. This study elucidated distinct shifts in rumen microbiota between ketosis and healthy dairy cows. As mentioned term “keto microbiota” previously, used to define a profile of gut microbiota moulded by a keto diet, perhaps we can refer to the specific microbial community in cows ketosis as cows “keto microbiota”. According to the results of microbiota analysis, the characteristics of rumen “keto microbiota” can be simply described as follows: the proportion of propionic acid producing bacteria decreased, the proportion of acetic acid and butyric acid producing bacteria increased, and the overall metabolic capacity declined.

In addition, the effects of rumen microbiota on mastitis have been reported. Ma et al. found that dysbiosis of intestinal microbiota could result in mastitis in mice [19]. Studies have shown that higher SCC are associated with differential microbial composition and function in the rumen, indicating the correlation between rumen microbiota and subclinical mastitis [1]. Some clinical trials have shown that oral probiotics supplementation could effectively control mastitis, suggesting that the mechanism of mastitis protection might be achieved via the host gut microbiota [20, 42]. It was reported that the pattern of faecal microbial community changes of mastitis cows was similar to that of the milk, characterized by a general increase in the mastitis pathogens and deprivation of *Lactobacillus* [20]. In the present study we found that the bacterial richness was extremely high in milk from ketosis cows, compared with healthy ones. Meantime, Venn diagram showed that a large number of OTUs shared by the rumen fluid and milk samples in ketosis cows. Interestingly, some of these common bacteria between rumen fluid and milk in ketosis cows changed with the same trend. In our study, both *F082* and *Thermomonas* were enriched in the milk and rumen fluid of ketosis cows with higher abundance than healthy cows. Furthermore, *Acinetobacter* and *UCG-005* were enriched in milk of healthy cows and reduced synchronously in milk and rumen fluid of ketosis cows. *F082*, an unclassified family belonging to *Bacteroidales*, possesses the second abundance only to *Prevotellaceae* and *Lachnospiraceae*. Although we do not know its specific function, we speculate that the *F082* bacterial taxa should also be capable of fermenting various
substrates in view of its high abundance and the multi-functional characteristics of Bacteroidetes. The genus Thermomonas is considered to be part of an abundant group of denitrification bacteria in ecosystems\cite{43}. Acinetobacter bacteria are ubiquitous in nature and usually cause infections in people and animals\cite{44}. It's strictly aerobic and nonfermenting characteristic may bring about reduction of abundance in rumen and milk in ketosis cows. Oscillospiraceae_UCG−005, as a bacterium that could not be cultured temporarily, with positive correlations with acetate and total short chain fatty acids (SCFAs) concentration in intestine of pig, might be related to the utilization of fiber diet\cite{45}. The relative abundance of Oscillospiraceae_UCG−005 decreased with Prevotella and Ruminococcus in rumen fluid of ketosis cows. Further analysis of the reasons for the simultaneous changes of these bacterial taxa in rumen fluid and milk from ketosis cows suggested the similar changes of metabolic substrates or the migration of bacteria between rumen and mammary gland.

Many studies suggested that some bacteria were indeed capable of dissemination from the gastrointestinal tract to mammary gland\cite{46, 47}, resulting in intramammary infection. Although the pathway and mode of microbial translocation are still obscure, general factors that can trigger bacterial translocation from the gut are host immune deficiencies and immunosuppression, disturbances in normal ecological balance of gut, mucosal barrier permeability, obstructive jaundice and stress\cite{48}. Abujamieh et. al demonstrated that increased intestinal permeability might play a role in the development of ketosis and possibly resulted in an increase of circulating lipopolysaccharide in plasma\cite{49}. Therefore, the immunosuppression caused by ketosis and NEB, postpartum stress, liver injury and systemic inflammatory induced by hyperketemia may be important prerequisites for bacterial migration. Meanwhile, changes of different kinds of bacteria suggested that ruminal bacteria might be trafficked to the mammary gland in a selective manner. In conclusion, the findings from this study shed new light on the mechanism of mastitis induced by ketosis and reveal the correlation between rumen microbiota and milk microbiome. Nevertheless, the specific function of differential bacteria and the detailed mechanisms on mastitis induced by dysbiosis of rumen microbiota remain unclear. Therefore, to clarify the effect of ruminal bacterial community on mastitis induced by ketosis, further research, including metabonomics and genomics, can be conducted.

**Conclusions**

Subclinical ketosis in dairy cows was accompanied by subclinical mastitis. The rumen harbored unique structures of bacterial profiles in ketosis cows, which were correlated with BHBA in plasma, SCC in milk and milk components. Furthermore, ketosis also led to alterations of milk microbiota, which showed a similar trend to the rumen microbiota. Ruminal microbial dysbiosis played an important role in the onset of ketosis and might contribute to the development of mastitis.

**Methods**

**Animals and Experimental Design**
Blood BHBA testing was performed on all cows at 7-14 days postpartum in a commercial dairy farm (Weifang, China). Blood BHBA ≥ 1.2 mmol/L was used as a diagnostic criterion for ketosis. All cows with significant clinical signs of ketosis and mastitis as well as other diseases were excluded. A total of 18 multiparous (average body weight, 554 ± 32 kg, parity ranged from 2 to 5, lactation days and weight similar) Holstein dairy cows were selected. Animals were divided into two groups according to the blood BHBA level, including 10 cows as disease group with blood BHBA ≥ 1.2 mmol/L, 8 cows as control group with blood BHBA < 1.2 mmol/L. In this experiment, SCC = 500,000 cells/mL and BHBA = 1.2 mmol/L was set as the threshold of mastitis and ketosis, respectively[50, 51]. The animals were milked three times a day and fed the same diet containing the special concentrate for perinatal period three times daily, and had free access to water. The full proposal was reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jilin University ethics committee, which approved the animal care and use permit license.

Sample Collection and Assay

Blood samples were collected from the coccygeal vein using a 5 mL disposable syringe within 3 h of the morning feeding, immediately before the cows were milked. BHBA was determined with a hand-held meter (Yicheng, Beijing, China) at cow side. The teats and teat orifices were disinfected with pieces of cotton wool soaked in 70% ethyl alcohol and discarded the first three handfuls of milk. Then milk samples were collected from four quarters of each cow, mixed and divided into two portions. One portion was collected and transferred into sterile 50 mL vials with potassium dichromate. The samples were used to detect the milk composition (MilkoscanTM FT1, FOSS, Denmark) and SCC (Fossomatic 5000, FOSS). Another portion was frozen in liquid nitrogen and kept in -80°C used to the 16S rRNA rDNA gene amplicon pyrosequencing. Rumen content of all the cows was collected using oral stomach tubes around midmorning, approximately 2 h after feeding[52], and then the samples were frozen in liquid nitrogen and kept in -80°C used to the 16S rRNA gene amplicon pyrosequencing.

DNA Extraction and Sequencing

Total genome DNA from rumen fluid and milk was extracted by a CTAB/SDS method. The DNA concentration and purity of each sample were detected by 1% agarose gels and the concentration of DNA was diluted to 1 ng/µL by sterile water. The 16S rRNA was amplified by specific primer (16S V4:515F-806R) with the barcode targeting the V4 region. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed with an equal volume of 1×loading buffer (containing SYB green) and subjected to electrophoresis on a 2% agarose gel for detection. PCR products were mixed in equidensity ratios, and the products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer’s instructions. The library quality was evaluated by a Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequence on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Analysis of Sequencing Data
Paired-end reads were merged using FLASH (V1.2.7)[53]. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIIME (V1.9.1) quality control process[54]. The tags were compared with the reference database (Silva database) using UCHIME algorithm to detect chimera sequences[55], and then the chimera sequences were removed to obtain the effective tags. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic unit (OTU). Venn diagrams was used to show the distribution of common and unique OTUs among different samples. Alpha diversity was applied in analyzing complexity of species diversity for a sample through 4 indices, including Chao 1, ACE, Shannon and Simpson. In addition, Principal coordinate analysis (PCoA) of weighted UniFrac analysis was used to evaluate the distance among the rumen fluid and milk from ketosis and control. The Nonmetric multidimensional scaling (NMDS) of Bray-Curitis dissimilarity were used to evaluate the distance of rumen fluid and milk of cows from ketosis and control. Linear discriminant analysis (LDA) effect size (LEfSe) was conducted to identify bacterial taxa differentially represented between ketosis and healthy cows in rumen fluid and milk samples, and significant differences were considered by a LDA score > 4 and $P < 0.05$ [56]. MetaStat analysis was also used to identify differential bacteria between ketosis and healthy cows in rumen fluid and milk samples. Finally, the spearman correlation analysis was employed to explore the relationship between the relative abundance of individual bacterial taxa at genus level and blood BHBA and milk components[57].

**Statistical Analysis**

Statistical analysis was conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA). All data are expressed as the means ± SEM. Differences between data were determined using one-way ANOVA (Dunnett’s t-test) and the two-tailed t-test. A $P < 0.05$ was considered to be statistically significant. Spearman correlation coefficients were calculated to evaluate the correlation between data. The correlation coefficients $r > 0$ means positive correlation, and $r < 0$ means negative correlation. A $P < 0.05$ was considered to be statistically significant.

**Abbreviations**

**NEB**: Negative Energy Balance  
**BHBA**: $\beta$-hydroxybutyrate  
**NEFA**: Non-esterified Fatty Acids  
**SCC**: Somatic Cell Count  
**NF-κB**: Nuclear factor-kappaB  
**VFAs**: Volatile Fatty Acids  
**SCFAs**: Short Chain Fatty Acids  
**OTUs**: Operational Taxonomic Units
PCoA: Principal Co-ordinates Analysis

NMDS: Non-metric Multidimensional Scaling

DMI: Dry Matter Intake

LEfSe: Linear Discriminant Analysis Effect Size

LDA: Linear Discriminant Analysis

Declarations

Ethics approval and consent to participate

The study proposal was reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jilin University ethics committee, which approved the animal care and use permit license, and complied with the Animal Protection Act and the Animal Welfare Guidelines of the World Animal Health Organization (WOAH, OIE).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files. The relevant datasets analyzed in the current study are available from the corresponding authors upon reasonable request.

Competing interests

None of the authors have a financial interest in any of the products, devices, or Materials mentioned in this manuscript. The authors declare that they have no conflicts of interest.

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Authors’ contributions

K X contributed to article writing, literature search, results evaluation. R M, S L, and Y W contributed to the sample selection and analysis. C Z contributed to literature search. X H performed the final revision of the article and expert opinions. N Z and Y F contributed to study design.

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**Figures**

**Figure 1**

**Comparation of blood BHBA, SCC and milk components between ketosis cows and healthy cows.** (A) Blood BHBA (mmol/L), (B) somatic cell count (SCC) (‘10000/mL), (C) milk protein (%), (D) lactose (%), (E) milk fat (%), (F) urea nitrogen (mg/dL) and (G) milk fat/protein ratio were compared between ketosis cows and healthy cows.
Figure 2

**Comparison of microbiota diversity and richness from rumen fluid between ketosis cows and healthy cows.** The microbiota richness in different groups was estimated in terms of chao 1 (A) and ace index (B). The microbiota diversity in different groups was estimated in terms of the shannon (C) and simpson index (D). KR and HR refer to rumen fluid samples in ketosis cows and healthy cows, respectively, while KM and HM refer to milk samples in ketosis cows and healthy cows, respectively. $P < 0.05$ indicates a significant difference between the different groups.
Figure 3

**Similarity of bacterial community composition in milk and rumen fluid between ketosis cows and healthy cows.** Principal coordinate analysis (PCoA) (A) of the weighted UniFrac metric and Non-metric multi-dimensional scaling (NMDS) (B) of weighted UniFrac distance matrix revealed a shift of community diversity in rumen and milk from healthy cows to ketosis cows.

Figure 4

**Relative abundance of bacterial community at phylum and genus levels between healthy and ketosis.** Relative abundance of top 10 phylum (A) in milk and rumen, top 10 genus (C) in rumen and top 10 genus (D) in milk are showed through different colors in different groups. The areas marked yellow represent the remaining bacterial communities except for the top10. The *Firmicutes to Bacteroidetes* ratio (B) was compared between ketosis cows and healthy cows ($P = 0.02$).

Figure 5

**The distribution of common and unique OTUs between rumen fluid and milk in ketosis and healthy cows.** Venn diagrams showed the distribution of common and unique OTUs between ketosis and healthy cows in rumen fluid (A) and milk (B), between rumen fluid and milk in healthy (C) and ketosis cows (D).

Figure 6

**Analysis of differential bacterial taxa in rumen fluid between ketosis and healthy cows.** Comparing differential bacterial community in ketosis cows with healthy cows, the heatmap based on MetaStat analysis showed the differential species at genus (A) in rumen. The histogram of LDA value distribution (B) with LDA score > 4.0 and cladogram (C) showed differential bacterial community in rumen between ketosis cows and healthy cows.

Figure 7

**Analysis of differential bacterial taxa in milk between ketosis and healthy cows.** The histogram of LDA value distribution (A) with LDA score > 2.5 and cladogram (B) showed differential bacterial community in milk between ketosis cows and healthy cows. Comparing differential bacterial community in ketosis cows with healthy cows, MetaStat analysis showed the differential species at genus in milk (C).
**Figure 8**

**Correlation Between Rumen Microflora and Blood BHBA, SCC, and Milk Components.** Through spearman correlation analysis, correlations between the blood BHBA and milk components and bacterial taxa with top 35 relative abundance in genus level are illustrated in a heatmap. The scale colors denote whether the correlation is positive (closer to 1, red squares) or negative (closer to −1, blue squares) between the genera and the parameters.

**Supplementary Files**
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