Integrated meta-omics analyses reveal a role of ruminal microorganisms in ketone body accumulation and ketosis in lactating dairy cows

Qiuju Wang,1,2* Yizhe Cui,1* Nagaraju Indugu,3* Juan J. Loor,4 Qianming Jiang,4 Zhongtang Yu,5 Linda Baker,3 Dipti Pitta,3 Zhaoju Deng,6 and Chuang Xu6†

1College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, No. 2 Xinyang Road, New Development District, Daqing, Heilongjiang, China 163319
2Key Laboratory of Efficient Utilization of Feed Resources and Nutrition Manipulation in Cold Region of Heilongjiang Province, China 163319
3Department of Clinical Studies, School of Veterinary Medicine, New Bolton Center, University of Pennsylvania, Kennett Square 19348
4Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana 61801
5Department of Animal Sciences, The Ohio State University, Columbus 43210
6College of Veterinary Medicine, China Agricultural University, Beijing, China 100083

ABSTRACT

The extent to which a nutrition-related disorder such as ketosis alters the ruminal microbiota or whether microbiota composition is related to ketosis and potential associations with host metabolism is unknown. We aimed to evaluate variations occurring in the ruminal microbiota of ketotic and nonketotic cows in the early postpartum period, and how those changes may affect the risk of developing the disease. Data on milk yield, dry matter intake (DMI), body condition score, and blood β-hydroxybutyrate (BHB) concentrations at 21 d postpartum were used to select 27 cows, which were assigned (n = 9 per group) to a clinical ketotic (CK, 4.10 ± 0.72 mmol BHB/L, DMI 11.61 ± 0.49 kg/d, ruminal pH 7.55 ± 0.07), subclinical ketotic (SK, 1.36 ± 0.12 mmol BHB/L, DMI 15.24 ± 0.34 kg/d, ruminal pH 7.58 ± 0.08), or control (NK, 0.88 ± 0.14 mmol BHB/L, DMI 16.74 ± 0.67/d, ruminal pH 7.61 ± 0.03) group. Cows averaged 3.6 ± 0.5 lactations and a body condition score of 3.11 ± 0.34 at the time of sampling. After blood serum collection for metabolomics analysis (1H nuclear magnetic resonance spectra), 150 mL of ruminal digesta was collected from each cow using an esophageal tube, paired-end (2 × 300 bp) sequencing of isolated DNA from ruminal digesta was performed via Illumina MiSeq, and sequencing data were analyzed using QIIME2 (v 2020.6) to measure the ruminal microbiota composition and relative abundance. Spearman correlation coefficients were used to evaluate relationships between relative abundance of bacterial genera and concentrations of serum metabolites. There were more than 200 genera, with approximately 30 being significant between NK and CK cows. Succinivibrionaceae UCG 1 taxa decreased in CK compared with NK cows. Christensenellaceae (Spearman correlation coefficient = 0.6), Ruminococcaceae (Spearman correlation coefficient = 0.6), Lachnospiraceae (Spearman correlation coefficient = 0.5), and Prevotellaceae (Spearman correlation coefficient = 0.6) genera were more abundant in the CK group and were highly positively correlated with plasma BHB. Metagenomic analysis indicated a high abundance of predicted functions related to metabolism (37.7%), genetic information processing (33.4%), and Brite hierarchies (16.3%) in the CK group. The 2 most important metabolic pathways for butyrate and propionate production were enriched in CK cows, suggesting increased production of acetyl coenzyme A and butyrate and decreased production of propionate. Overall, the combined data suggested that microbial populations may be related to ketosis by affecting short-chain fatty acid metabolism and BHB accumulation even in cows with adequate feed intake in the early postpartum period.

Key words: lactation, metagenomics, metabolomics, transition period

INTRODUCTION

The periparturial or “transition” period in dairy cows refers to the time from 3 wk precalving to 3 wk postcalving and constitutes the most challenging physiological state in the life cycle of dairy cows (Bell, 1995). During this period, the energy required by the mammary gland to initiate and maintain lactation exceeds that of dietary energy intake, resulting in a state of negative energy balance characterized by lower blood glucose concentrations and an increase in body fat mobilization (Xu et al., 2020). Ketosis is characterized by an increase of ketone bodies in the peripheral blood circulation as a result
of negative energy balance at the onset of lactation and the subsequent mobilization of adipose tissue stores that fill the liver with fatty acids (Soares et al., 2021). Oxidation of fatty acids to acetyl-CoA in the liver increases the use of this intermediate toward ketogenesis, which could lead to production of acetone, acetoacetic acid, and BHB, collectively referred to as ketone bodies (Bell, 1995). A state of subclinical ketosis (SK) pertains to the accumulation of ketone bodies in the blood, urine, and milk without any clinical signs. Subclinical ketosis can advance to clinical ketosis (CK) that is evidenced by high concentrations of ketone bodies in body fluids and that is accompanied by loss of appetite, decreased milk production, rapid weight loss, and dry feces (Gordon et al., 2013). Regardless of the type of ketosis, BHB concentrations are considered the generally accepted standard for ketosis diagnosis, with concentrations ranging from 1.2 to 1.4 mmol/L classified as SK (Suthar et al., 2013) and $>3$ mmol/L as CK (Oetzel et al., 2007).

Although extensive research has investigated the different factors that may predispose prepartum dairy cows to ketosis or the systemic changes that lead to ketosis, the role that ruminal microbiota and their genomes play in the risk of SK or CK is unknown. For instance, it remains to be determined whether there are specific ruminal microbes that may be the cause or have a critical role in the incidence of ketosis.

Ruminants harbor a complex microbial community (or microbiota) comprising bacteria, protozoa, fungi, and archaea in the rumen that collectively digest and ferment carbohydrate into VFA, and many other compounds, and synthesize microbial proteins that are then used by the host to make milk and meat (Gebreyesus et al., 2020). The ruminal microbiota is dynamic in nature and changes with breed, dietary composition, feed intake level, physiological status, health status, environmental changes, and geographic location (Kim et al., 2020). Several studies have investigated the ruminal microbiota of dairy cows during the transition period (Pitta et al., 2014, 2016; Lima et al., 2015; Zhu et al., 2017) and documented microbial shifts during this transition phase. However, it is largely unknown how the relative microbial abundance and composition shift in the rumen of cows that transition smoothly compared with cows that experience ketosis.

Recent studies on periparturient cows have revealed that the ruminal microbiota are positively correlated with milk yield and milk composition—that is, feed-efficient cows have unique profiles of ruminal microbiota, some of which are correlated with concentrations of milk fatty acids; and specialist bacteria have the potential to enhance rumen fermentative digestion of feedstuffs to support higher milk yields (Indugu et al., 2017; Seshadri et al., 2018; Zhang et al., 2022). Morgavi et al. (2015) concluded in a review that the ruminal microbiota could indirectly affect host metabolism, including protein synthesis. Recently, when tracking dairy cows for postpartum complications on commercial farms, we noted that even under the same nutritional management, some cows were more susceptible to ketosis than others in the postpartum period (Kaplan-Shabtai et al., 2021). Considering the host specificity of ruminal microbes and VFA (Kaplan-Shabtai et al., 2021), compared with cows that do not experience ketosis, we speculated that the onset of CK and decrease in performance after parturition might be associated with ruminal microbes.

The objective of this study was to evaluate what, if any, variations occur and potential associations of ruminal microbiota among SK, CK, and healthy dairy cows, and how the ruminal microbiota might correlate to ketosis.

**MATERIALS AND METHODS**

Animal care and experimental procedures were approved by the animal welfare and ethics committee of Heilongjiang Bayi Agricultural University (Daqing, China).

**Animals, Sampling, and Physiological Parameters**

Cows used in the present study were selected from a 700-head commercial farm. Data on milk yield, DMI, BCS, rumen pH, and blood BHB concentration [control (NK), BHB < 1.2 mmol/L; SK, 2.0 mmol/L > BHB > 1.2 mmol/L; CK, BHB > 3.0 mmol/L] at 21 d postpartum were used to select 27 cows, some of which did not have a clinical disease and some of which had ketosis. These cows were grouped ($n = 9$ per group) into the following: 9 cows in the CK group with a serum BHB concentration of $4.10 \pm 0.72$ mmol/L, DMI $11.61 \pm 0.49$ kg/d, ruminal pH $7.55 \pm 0.07$; 9 SK cows with a serum BHB concentration of $1.04 \pm 0.12$ mmol/L, DMI $15.24 \pm 0.34$ kg/d, ruminal pH $7.58 \pm 0.08$; and 9 NK cows with a serum BHB concentration of $0.88 \pm 0.14$ mmol/L, DMI $16.74 \pm 0.67$ kg/d, ruminal pH $7.61 \pm 0.03$. Feeders in the farm measured DMI for each cow every day. The DMI was measured the day before sampling. The cows had similar age ($4.63 \pm 0.49$ yr), parity ($3.56 \pm 0.51$), and BCS ($3.11 \pm 0.34$) at the time of sampling (Supplemental Table S1, https://doi.org/10.6084/m9.figshare.21687572.v1, Cui, 2022).

Cows were fed a commercial diet thrice daily at 6:00 a.m., 2:00 p.m., and 9:00 p.m. and were milked thrice daily at 5:00 a.m., 2:00 p.m., and 9:00 p.m. and were milked thrice daily at 5:00 a.m., 2:00 p.m., and 9:00 p.m. and were milked thrice daily at 5:00 a.m., 2:00 p.m., and 9:00 p.m. and were milked thrice daily at 5:00 a.m., 2:00 p.m., and 9:00 p.m. and were milked thrice daily at 5:00 a.m., 2:00 p.m., and 9:00 p.m.
Blood samples were collected from the tail vein at 21 d postpartum in the same year in the morning before feeding into tubes containing a polymer gel for serum separation. After centrifugation for 10 min at 3,000 \(\times\) g at 4°C, serum samples were collected to measure chemical parameters such as BHB with a portable blood ketone detector (Wu et al., 2018). Following blood sampling, 150 mL of ruminal fluid was collected from each cow using an esophageal tube inserted into the rumen, with the first 200 mL of the initial saliva mixed with ruminal fluid being disposed. All ruminal liquid samples were filtered through sterile gauze and then transferred into cryopreservation tubes, which were immediately placed in liquid nitrogen and transported to the laboratory within 2 h and then stored at −80°C.

### Analysis of Serum Metabolome

The \(^1\)H nuclear magnetic resonance spectra of all samples were determined with a Varian Unity Inova 600 MHz superconducting nuclear magnetic resonance spectrometer. The Carr-Purcell-Meiboom-Gill sequence was used to suppress broad resonance signals such as those corresponding to the proteins in the serum (Goldoni et al., 2016). All free induction decays were zero-filled to 64 K points and multiplied by an exponential function with a 1.0-Hz line-broadening factor before Fourier transformation. The relaxation delay was 2.1 s, the spectral width was 8,000 Hz, the temperature was 298.2 K, the total echo time was 100 ms, and the number of scans was 96. To improve the signal-to-noise ratio, the spectra of all samples were multiplied by the broadening factor. After that, a 1-Hz window function was performed. The \(^1\)H nuclear magnetic resonance spectrum and baseline corrections were processed manually using TopSpin (V3.0 Bruker Biospin).

### DNA Extraction, Metataxonomic and Metagenomic Sequencing, and Data Processing

Total genomic DNA was extracted from ruminal fluid samples using the repeat bead-beating plus column method (Yu and Morrison, 2004) with the PureLink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific). The V3–V4 region of the 16S rRNA genes was PCR amplified using the primers 338F (5′-barcode-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACH VGGGTWTCT-AAT-3′), both of which had unique barcodes for multiplexing. The PCR products were checked visually using agarose (2%) gel electrophoresis purified using a DNA gel extraction kit (Axygen Inc., catalog no. AP-GX-50). The purified PCR products were quantified fluorospectrometrically using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Ltd. catalog no. P11496). Sequencing libraries from the PCR products were prepared using the TruSeq Nano DNA LT Library Prep Kit (Illumina). The libraries were then quantified using the Quanti-IT PicoGreen dsDNA Assay Kit using the Quantifluor fluorescence quantitative system (Promega). Amplicon libraries were mixed in an equal molar ratio and paired-end (2 × 300 bp) sequenced on an Illumina MiSeq system by Shanghai Personalbio (Shanghai, China).

### Bioinformatics and Statistical Analysis

The raw amplicon sequencing data were processed using QIIME2 (v 2020.6). Briefly, the paired-end sequencing reads were merged using the VSEARCH wrapper with the default setting (Bolyen et al., 2019). The merged sequences were quality filtered using the quality-filter q-score method with the default settings and then grouped into unique amplicon sequence variants (ASV) using the built-in deblur plugin (Sokolovsky et al., 2017). The MAFFT (Katoh and Standley, 2013) program was used to generate multiple sequence alignment and to filter out highly variable positions with the default settings. A phylogenetic tree was constructed using FastTree 2 (Price et al., 2010) with default settings. Singletons (unique sequences that occurred only once) were removed from the representative sequences of ASV and were rarified to 5,755 sequences per sample. Alpha diversity (observed ASV and Shannon diversity) and \(\beta\) diversity (weighted and unweighted UniFrac distances) were computed using the “qiime diversity” plugin. A pretrained Naive Bayes classifier trained on the SILVA database (v132; Quast et al., 2013) for the 16S rRNA genes spanning the V3–V4 region was used for taxonomic classification. The measured \(\alpha\) diversity indices were statistically compared among cow groups using the Wilcoxon/Kruskal-Wallis rank sum test. A nonparametric permutational multivariate ANOVA test (Anderson et al., 2001) implemented in the vegan package for R was used to compare the overall ruminal microbiota among cow groups. To determine whether the relative abundance of individual bacterial taxa was significantly different among cow groups, we conducted the Wilcoxon/Kruskal-Wallis rank sum test. We also analyzed individual bacterial taxonomy using analysis of composition of microbiomes. The Spearman correlation coefficients (Schober et al., 2018) were calculated to evaluate relationships with their relative abundance between bacterial genera and serum BHB.

Raw shotgun metagenomic sequences were subjected to quality trimming using Trimmomatic (0.36) (Bolger et al., 2014) according to the following parameters: starting from either end of the sequences, bases were trimmed off if their Phred quality score was <3 or if
they appeared as N; bases were trimmed off if their average Phred quality score was <15 over a 4-base sliding window; and sequences were removed if they were shorter than 36 bases in length. Reads matching the host genome (ARS-UCD1.2/bosTau9) were identified and removed using Bowtie2 (v2.2.7; Langmead and Salzberg, 2012) with parameters set by the flag “--very sensitive local–un-conc.” The quality-filtered sequences were annotated to functional categories against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with Diamond software (Kanehisa and Goto, 2000). Kyoto Encyclopedia of Genes and Genomes is a knowledge base for systematic analysis of gene functions, linking genomic information with higher-order functional information. The genomic information is stored in the GENES database, which is a collection of gene catalogs for all the completely sequenced genomes and some partial genomes with up-to-date annotation of gene functions. The higher-order functional information is stored in the PATHWAY database, which contains graphical representations of cellular processes such as metabolism, membrane transport, signal transduction, and cell cycle. The PATHWAY database is supplemented by a set of ortholog group tables for the information about conserved subpathways (pathway motifs), which are often encoded by positionally coupled genes on the chromosome and which are especially useful in predicting gene functions. A third database in KEGG is LIGAND, which contains information about chemical compounds, enzymes, and enzymatic reactions. Kyoto Encyclopedia of Genes and Genomes provides Java graphics tools for browsing genome maps, comparing 2 genome maps, and manipulating expression maps, as well as computational tools for sequence comparison, graph comparison, and path computation. The KEGG databases are updated regularly and are freely available (http://www.genome.ad.jp/kegg/).

For taxonomic classification, the quality-filtered reads were mapped to a low-complexity masked database of bacterial, archaeal, viral, fungal, and protozoal complete genomes from the National Center for Biotechnology Information (downloaded on October 22, 2020) using Kraken (Wood et al., 2019) and the abundances of individual species were computed using Bracken (Lu and Salzberg, 2020). We conducted principal coordinates analysis based on the Bray–Curtis dissimilarity of both the taxonomic and functional profiles using the vegan package in R, and the principal coordinates analysis results were tested for statistical significance using a permutational multivariate ANOVA test. We used generalized linear models available in R to determine whether the normalized abundances (counts per million) of individual functional categories were significantly different among cow groups. A P-value < 0.05 means significant difference.

RESULTS

Characterization of Animal Phenotypes

Data on milk yield, BCS, DMI, and blood biochemical indices were obtained from 715 dairy cows in the same year. From that cohort, 27 cows with different blood BHB concentrations were selected. These cows all calved in the month of September, did not experience heat stress with the average temperature at 8°C at night and 20°C during the day, were free of other clinical diseases, and averaged 3 to 4 parities. This group included 9 cows with blood BHB concentrations > 1.2 mmol/L but < 3.0 mmol/L (averaged 1.36 ± 0.12 mmol/L) and deemed as SK, 9 cows with BHB > 3.0 mmol/L (averaged 4.1 ± 0.72 mmol/L) and deemed as CK, and 9 cows with BHB < 1.2 mmol/L (averaged 0.88 ± 0.14 mmol/L) deemed as NK. From these cows, ruminal contents and blood samples were collected for metagenome and serum BHB analyses. The BHB concentration (the accepted standard for ketosis diagnosis) differed among the 3 groups (Supplemental Table S1, P = 0.001), with concentrations being greatest in the CK cows (4.10 ± 0.72 mmol/L) followed by the SK cows (1.36 ± 0.12 mmol/L) and NK cows (0.88 ± 0.14 mmol/L).

Determination of Microbial Sequences

Metataxonomic sequencing generated a total of 1,289,863 raw reads of 16S rRNA gene amplicons from a total of 27 samples. After quality filtering and denoising, a total of 274,056 (5,755 to 15,038 per sample) ASV were obtained. For diversity analysis, we rarified ASV at a sequencing depth of 5,755 sequences for a total of 27 samples. After quality filtering and denoising, a total of 1,289,863 raw reads of 16S rRNA gene amplicons from a total of 27 samples. After quality filtering and denoising, a total of 274,056 (5,755 to 15,038 per sample) ASV were obtained. For diversity analysis, we rarified ASV at a sequencing depth of 5,755 sequences for sample. Representative sequences from individual ASV were taxonomically assigned to 17 bacterial phyla and 219 bacterial genera.

Metagenomic sequencing yielded a total of 382,680,654 paired-end reads from 27 metagenomic samples. The average number of sequences per sample was 38,349,106.67 (minimum: 31,972,553; maximum: 42,162,208).

Differences in Ruminal Microbiota Correspond to the 3 Ketotic States

No difference (P > 0.05) in the number of observed species (ASV) was noted between NK and SK, but CK tended to have a higher (P = 0.065; Wilcoxon test)
observed richness than NK (Figure 1A). Significant differences in the Shannon diversity index (Figure 1B) were noted among all 3 cow groups ($P < 0.05$; Kruskal-Wallis test), with CK and SK being higher than NK. Further, pairwise comparisons using the Wilcoxon test showed that CK was different ($P < 0.05$) from both NK and SK, but no difference was observed between NK and SK.

Beta diversity analysis was used to evaluate the difference of the overall ruminal microbiota among the 3 cow groups. Principal coordinates analysis based on either unweighted or weighted UniFrac distances revealed no significant differences among the 3 cow groups; however, the ruminal microbiota of the CK cows were clustered tightly, whereas those of the NK cows were scattered along the first 2 principal coordinates (Supplemental Figure S1, https://doi.org/10.6084/m9.figshare.21687572.v1, Cui, 2022).

The dominant bacterial phyla were Bacteroidetes (49.10% ± 1.704%) and Firmicutes (36.30% ± 2.079%), followed by Proteobacteria (8.04% ± 2.796%), Fibrobacteres (1.73% ± 1.18%), Spirochaetes (1.39% ± 1.28%), and Tenericutes (0.95% ± 0.53%) (Figure 2; Supplemental Table S2, https://doi.org/10.6084/m9.figshare.21687572.v1, Cui, 2022). More than 200 genera were detected in this study, and 39 had different ($P < 0.05$) relative abundance among the 3 cow groups, with 6 genera differing between NK and SK and 30 genera differing between NK and CK. Notably, 24 genera had different relative abundance between SK and CK, indicating that the microbial profiles of the CK cows could be distinct from those of the SK and NK cows.

Only taxa belonging to Succinivibrionaceae UCG 1 differed among the 3 cow groups, with a decrease in the CK cows compared with the NK cows (NK: 10.67 ± 4.33 vs. CK: 0.04 ± 0.02). Pairwise comparisons among the 3 cow groups revealed more significant differences (Supplemental Table S2). Several genera of Bacteroidetes, such as Uncultured Prevotellaceae, Rikenellaceae, and Bacteroidales, and several genera of Firmicutes,

![Figure 1.](https://example.com/figure1.png)

**Figure 1.** The (A) richness [numbers of observed amplicon sequence variants (ASV), $P = 0.066$] and (B) Shannon diversity index ($P = 0.015$) of the rumen microbiota in the control (NK), subclinical ketosis (SK), and clinical ketosis (CK) cow groups as determined using metataxonomics. Line: the median line divides the boxes into 75% and 25%. Boxes: the height of the box represents the range of sample data and reflects the fluctuation degree of data within the group. Whiskers: maximum values (top part) and minimum values (bottom part) except outliers. Dots: the data location of the individual sample in the group under this index.

![Figure 2.](https://example.com/figure2.png)

**Figure 2.** Stacked bar plot depicting the relative abundances of the bacterial phyla detected using metataxonomics in the control (NK), subclinical ketosis (SK), and clinical ketosis (CK) cow groups. Relative abundances of Bacteroidetes ($P = 0.037$) and Tenericutes ($P = 0.023$) were significantly lower in NK than CK, and only Proteobacteria ($P = 0.017$) was significantly higher in NK than in CK.
including *Christensenellaceae*, *Lachnospiraceae*, and *Ruminococaceae*, were more abundant in the CK cows than in the NK cows. On the contrary, several genera, particularly *Prevotella 7*, *Ruminococcus 2*, Selenomonas, Unclassified Veillonellaceae, and *Succinivibrionaceae*, were less abundant in the CK cows compared with NK cows.

### Relationships Between Serum Metabolites and Relative Abundance of Ruminal Bacteria

Liquid chromatography–mass spectrometry detected 30 metabolites in the serum of the 3 groups. Six of these metabolites, including BHB, acetoacetate, acetone, acetaldehyde, acetyl glycoprotein, and unassigned resonance I, had a higher relative abundance in the SK and CK cows than the NK cows (*P* < 0.05; Supplemental Table S3, https://doi.org/10.6084/m9.figshare.21687572.v1, Cui, 2022). In contrast, 13 metabolites had lower relative concentration in the SK and CK cows than the NK cows, and these were glucose, lactate, citrate, formate, 1- and 3-methylhistidine, alanine, glutamate, glutamine, lysine, phenylalanine, proline, and tyrosine (*P* < 0.05; Supplemental Table S3).

Spearman’s rank correlation analysis revealed that the relative abundances of a total of 181 bacterial genera (or equivalent taxa) across all 3 cow groups were either positively or negatively correlated with the relative concentration of one or more serum metabolites (Supplemental Table S4, https://doi.org/10.6084/m9.figshare.21687572.v1, Cui, 2022). Twenty-nine bacterial genera that differed in relative abundance among the 3 cow groups exhibited positive or negative correlation with 1 or more serum metabolites (Figure 3). Several genera that were significantly more abundant in the CK than NK cows, including *Christensenellaceae*, *Ruminococaceae*, *Lachnospiraceae*, and *Prevotellaceae*, were highly positively correlated with BHB. However, *Succinivibrionaceae*, which was significantly less abundant in the CK than NK cows, was strongly negatively correlated with BHB. *Christensenellaceae*, *Ruminococaceae*, *Prevotellaceae*, and *Succinivibrionaceae* also had a correlation with myo-inositol, a lipotropic factor, but in the opposite direction of the correlation with BHB. These are the only 2 metabolites that had a correlation with any bacterial genera, whereas other lipogenic factors such as choline or the muscle protein breakdown markers 1-methylhistidine and 3-methylhistidine were not positively correlated with any bacterial genera. Among the amino acids, tyrosine and phenylalanine were positively correlated with *Succinivibrionaceae*, whereas glutamine was negatively correlated with *Succinivibrionaceae*. Tyrosine had a positive correlation with *Ruminococaceae* and *Lachnospiraceae*, and valine and U2 had a positive correlation with *Christensenellaceae*, *Ruminococaceae*, and *Lachnospiraceae*. Glucose-a had a positive correlation with *Ruminococaceae* and a negative correlation with *Succinivibrionaceae*.

### Microbial Metabolic Pathways and Ketotic State

At level 1, KEGG pathways related to metabolism (37.7%), genetic information processing (33.4%), and Brite hierarchies (16.3%) were more abundant in the CK group (Figure 4a). At the KEGG level 2, a total of 35 gene families were identified in all the samples. Of these 35 gene families (Figure 4b), the majority belonged to translation (28.5%), carbohydrate metabolism (14.1%; metabolism), protein families: genetic information processing (11.6%; Brite hierarchies), nucleotide metabolism (7.41%; metabolism), and amino acid metabolism (6.0%; metabolism). No differences in any of the detected KEGG pathways, at either level 1 or level 2, were noted among the 3 cow groups (*P* > 0.05).

Ketone bodies originate from rumen fermentation products of butyrate and tissue metabolism, primarily to BHB; thus, we compared the 2 most important metabolic pathways for butyrate production (Figure 5). There was no statistical difference for genes involved in propionyl-CoA production among the 3 groups. However, the genes of ruminal bacteria encoding the enzymes involved in acetyl-CoA (EC: 1.2.7.1) production and butyryl-CoA (EC: 1.3.8.1) production were significantly higher in the CK than NK cows (*P* < 0.001; generalized linear models), suggesting that the production of acetyl-CoA and subsequently butyrate might be higher in the ruminal bacteria of CK compared with NK cows.

We also compared the 2 most important metabolic pathways for propionate production via the succinate pathway (Figure 6). The 3 genes encoding enzymes identified in this study involved in succinyl-CoA (EC: 6.2.1.5) production and propanoyl-CoA (EC: 1.3.8.7) production were less abundant in the CK than NK cows (*P* < 0.001; generalized linear models), supporting the hypothesis that less propionate via the succinate pathway might have been produced in the CK compared with NK cows.

### Discussion

Although it is well known that ruminal microorganisms are essential for the survival and productive efficiency of ruminants, it is still unknown what, if any, role these microbes might play in the occurrence of ketosis. In the present study, we used a multi-omics approach...
including metataxonomics, metagenomics, and host metabolomics that allowed us to examine the ruminal microbiota, their functions, and host serum metabolites that are associated with the degree of ketosis (i.e., nil, subclinical, or clinical ketosis) after parturition.

Although all the cows in the present study were fed the same commercial diet, ketotic cows consumed less DM and produced less milk, both of which are commonly seen during SARA, another common disorder of dairy cows. Unlike ketosis, however, SARA is primarily associated with the feeding of highly fermentable diets. Because of this, in the present study we measured ruminal pH and selected cows undergoing ketosis, but not SARA. Thus, the increased richness and diversity of the ruminal microbiota with ketosis were especially noteworthy—that is, this disorder can affect the ruminal microbiota by decreasing DMI without inducing SARA. It was reported that bacterial richness and diversity could be indicators of the functionality of the microbiota (Elolimy et al., 2018). Several biotic and nonbiotic factors can influence bacterial richness and diversity, and a reduction in these metrics was associated with altered metabolic processes such as milk fat depression in dairy cattle (Pitta et al., 2020). Importantly, richness and diversity characterized the ruminal microbiota of efficient dairy animals, and this was attributed to the abundance of specific microbial populations associated with simpler metabolic pathways but capable of harnessing more energy that is then used by the host (Shabat et al., 2016). In contrast, inefficient animals have a greater diversity that can use diverse substrates, leading to the formation of several metabolites that may not be completely used by the host (Shabat et al., 2016).

As noted in NK cows, the lower richness and diversity in the ruminal microbiota were analogous to those

Figure 3. Spearman’s rank correlation between relative sequence abundances of bacterial genera and serum metabolites in all the dairy cows. The colors and the intensity depict the direction and strength of the correlation, as shown by the bar to the right.
of efficient dairy cows, and an increased richness and diversity of ruminal microbiota structure and function lead to an increased microbial metabolome, which predisposes dairy cows to ketosis. Furthermore, the gradual increase in richness and diversity from the NK to the CK cows, with the SK cows being intermediate.

Figure 4. Composition of the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene families as detected using metataxonomics among the control (NK), subclinical ketosis (SK), and clinical ketosis (CK) cow groups. (A) KEGG pathways at level 1; (B) KEGG pathways at level 2. Panel C represents different KEGG pathways with different colors corresponding to the vertical colors in panel B. Error bars represent standard divisions among the NK, SK, and CK cow groups of the same index.
and having greater variation, also reflected a gradual shift in both the structure and function of the ruminal microbiota.

Ketosis status was accompanied by substantial changes in the bacterial taxa in the rumen. At the phylum level, compared with NK cows, both Bacteroidetes and Firmicutes had higher relative abundances in CK cows, followed by the SK cows. An increased species richness of Bacteroidetes was observed in the CK cows compared with the NK cows, but only a few genera differed in relative abundances among the 3 cow groups. Within Firmicutes, Lachnospiraceae and Ruminococcaceae were increased in the CK cows compared with the NK cows. Based on analysis of composition of microbiomes, we found that only Succinivibrionaceae, a family of Proteobacteria, substantially decreased its abundances in the CK cows compared with the NK cows. A greater abundance of Succinivibrionaceae was positively correlated with phenotypic traits such as higher feed efficiency and lower methane emissions (Pope et al., 2011). Species of this family can produce succinate, a precursor of propionate production, and a substantial reduction of this family in the CK cows can be indicative of suppression of fermentation pathways, leading to propionate formation therein.

Correlation analysis between ruminal bacteria and serum metabolites provided new insights into the role of rumen microbes in the incidence of ketosis. Anaeroplasma, Comamonas, uncultured bacteria from Firmicutes, uncultured genera UCG-010 and UCG-011, Ruminococcaceae NK4A214, Papillibacter, Lachnospiraceae XPB1014, Family XIII AD3011, Christensenellaceae R-7, Anaerovorax, and RFN46 are some lineages positively associated with BHB. Furthermore, these were the genera that increased significantly in the CK and the SK cows compared with the NK cows.

Recent work has provided biological evidence to associate ruminal microbiota with metabolites and phenotypes (Li et al., 2016). Acetate, propionate, and butyrate produced by the ruminal microbiota can be metabolized by the animal to furnish approximately 70% of their energy requirement (Eom et al., 2021). The application of serum metabolomics in the present study allowed for establishing biological associations between rumen microbes and serum metabolites. Notably, the enrichment of the microbial enzyme acetyl-CoA acyltransferase in the CK group was associated with a higher serum concentration of BHB, suggesting a potential contribution of some microbes that predominate during ketosis to the production of acetyl-CoA and the accumulation of ketone bodies, which are then absorbed into the systemic circulation of the cow (Annika and Alexander, 2013).

**CONCLUSIONS**

Overall, a unique ruminal microbiota corresponded to clinical ketosis, underscoring a biological association between this disease and rumen bacteria. Ketosis was associated with changes in rumen bacterial communi-
ties at the phyla and genus levels. Christensenellaceae, Ruminococcaceae, Lachnospiraceae, and Prevotellaceae were enriched in ketotic cows and may be associated with the production of BHB. This study also provided evidence that butyrate synthesis increases and contributes to the production of BHB. These findings provide foundational information for the distinct patterns in the ruminal microbiota that characterize ketotic and nonketotic lactating cows. In the future, in-depth metagenomic and metatranscriptomic analyses of ruminal microbiota on a larger number of cows integrated with metabolomics of ruminal contents and serum samples among ketotic, subclinical, and healthy cows will be required to understand the regulatory role of microbiota in the incidence of ketosis. Taken together, alterations in the ruminal microbiota, serum metabolome, and correlation analysis suggest that microbiota profiles contribute to ketosis.

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ORCIDS

Quuju Wang  https://orcid.org/0000-0002-9927-5412
Yizhe Cui  https://orcid.org/0000-0002-7877-8328
Nagaraju Indugu  https://orcid.org/0000-0001-9424-2155
Juan J. Loor  https://orcid.org/0000-0003-1586-4365
Qianming Jiang  https://orcid.org/0000-0001-9522-4856
Zhongtang Yu  https://orcid.org/0000-0002-6165-8522
Dipti Pitta  https://orcid.org/0000-0002-3102-9119
Chuang Xu  https://orcid.org/0000-0002-0377-1439