Potential Role of the Rumen Microbiome in Modulating Milk Protein and Fat in Dairy Cow Using Microgenomic Sequencing

Xin Wu  
China Agricultural University

Peng Peng  
China Agricultural University

Yanan Liu  
China Agricultural University

Bo Han  
China Agricultural University

Dongxiao Sun (✉ sundx@cau.edu.cn)  
China Agricultural University  https://orcid.org/0000-0002-7512-5946

Research

Keywords: Dairy cattle, Milk protein, Milk fat, Metagenome sequencing, Rumen microbiome

DOI: https://doi.org/10.21203/rs.3.rs-45889/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background

The rumen is the main digestive and absorption organ of dairy cows that contains abundant microorganisms and effectively utilizes human-indigestible plant mass. Investigation on microbiome in the rumen from lactating dairy cows using metagenomic sequencing is reasonable for identifying ruminal microorganisms that contribute to milk composition traits.

Results

We used the Illumina HiSeq platform to generate the rumen microbiome of the six lactating Holstein cows with extremely high and low milk protein and fat percentages (high and low groups of PP and FP). In total, 6977 microorganism species were detected in which Bacteroidetes (51.4%) and Prevotella (38.48%) was the most predominant phylum and genus, respectively. Between high and low groups, we observed significantly differential microorganism abundances in genus and species levels. By performing LEfSe and Metastats analyses, we identified 38 top abundant species displaying differential richness between two groups in common (LDA > 3, \( p < 0.05, q = 0.037 \sim 0.048 \)), in which Prevotella accounted for 68.8% of the species with higher abundance in high group. Function annotation with KEGG, eggnog and CAZy databases showed the species with significantly higher abundance in high group were enriched in carbohydrate, amino acid, pyruvate, insulin and lipid metabolism and transportation, indicating their higher capability of digesting feed and subsequently providing substrate for milk composition synthesis in mammary gland. In addition, a kind of anaerobic fungi, Neocallimastix californiae, was identified in high group that could coexist with rumen microbes and promote cellulose digestion.

Conclusion

This study investigated the rumen microbiome in lactating Holstein cows using metagenomic sequencing. Significant differential bacterial richness were observed between the cows with extremely high and low PP and FP. Function annotation showed the abundant species in high group were involved in carbohydrate, amino acid, pyruvate, insulin and lipid metabolism and transportation, indicating the significant correlation between rumen microbiota and milk compositions formation in dairy cattle.

Introduction

Cow’s milk acts as an abundant source of high-quality proteins, minerals and trace elements and is generally considered balanced and nutritive foods, being frequently included as important components of a healthy diet [1]. The rumen is the main digestive and absorption organ of dairy cows and it can effectively use grass, food and non-protein nitrogen to produce milk [2-4]. The rumen contains abundant microorganisms, comprising bacteria, protozoa and fungi, and is like a large anaerobic fermenter [2, 5, 6],
which digested complex fibrous substrates into fermentable sugars, ultimately, were fermented by rumen bacteria and converted primarily into volatile fatty acids (VFA) [7]. Approximately 90% of the VFA produced in rumen are acetate, butyrate, propionate that are absorbed into the blood through the rumen wall and transported to the liver. Subsequently, the liver transports cholesterol to the mammary gland for lipid synthesis in the form of lipoproteins [8-14]. As for lactose synthesis, glucose from carbohydrate digestion in rumen and gluconeogenesis in liver was transported into the mammary gland through blood circulation and converted into lactose [18]. Amino acids produced by the breakdown of rumen microbiota are absorbed into the blood in small intestine and converted into free amino acids (FAAs) that are subsequently flowed into mammary gland where FAAs were synthesized into milk protein [19].

Metagenomics is an analysis of microbial communities at particular habitat by using high-throughput sequencing without necessary requirement of laboratory isolation and culture of individual strains [15, 16]. It has been widely used to study microbial diversity and metabolic capabilities of microbiome in different ecological niches, fermented food, waste-water treatment facilities, and gastrointestinal tracts in human and animals [17-23]. In dairy cattle, rumen microbes played a vital role in the decomposition of plant lignocellulosic matter [24]. Jewell et al. found the ruminal bacterial community is dynamic in terms of membership and diversity and specific members are associated with milk production efficiency over two lactation cycles [25]. Jami et al. reported that rumen microbial diversity were closely related to milk yield and composition in which the ratio of the Firmicutes to the Bacteroides was correlated with milk fat yield [26]. So far, most researches on rumen microbiota in dairy cows mainly focused on the phylum and genus of rumen bacteria and their effects on milk production, feed conversion ratio and methane emissions mainly through 16S rRNA sequencing. From species level, regulation roles of rumen microbiota on milk composition traits has been still under investigated in dairy cattle.

In the present study, the purpose was to investigate the rumen microorganism constitutions of the lactating Holstein cows who exhibited extremely high and low milk PP and FP with high-throughput metagenomics technology, and identify the microbiota with significantly differential abundance between the high and low groups of PP and FP. Further, functional enrichment analysis were performed to elucidate the potential correlation of rumen microbiome with milk protein and fat biosynthesis.

**Materials And Methods**

**Animals and sample collection**

We selected six healthy lactating Chinese Holstein cows from the Beijing Sanyuanlvhe Dairy Farming Center, which were in the almost same lactation period (230, 272, 230, 281, 233, and 196 days in milk of the second lactation, respectively) and were fed the same total mixed rations. The cows were chosen among more than 4000 lactating Holstein cows based on their monthly test-day milk PP and FP records for the first and second lactations, which were provided by the Dairy Data Center of China (https://www.holstein.org.cn/). The six cows were divided into two groups with extremes of the average phenotypic values for PP and FP: three cows (high group) had high PP (3.7%, 3.6 and 3.8%) and FP (3.9%,
4.3% and 4.5%); the other three cows (low group) showed low PP (3.0%, 3.1% and 3.2%) and FP (3.1%, 3.2% and 2.9%).

Rumen fluid sample was collected from the rumen of each cow within 15 minutes after slaughter in the same slaughterhouse. All samples were collected in the cryogenic vials and stored in liquid nitrogen for further analysis. All animal works were carried out in accordance with the approved guidelines for the care and use of experimental animals from the Ministry of Agriculture of China. The protocol was specially approved by the Animal Welfare Committee of China Agricultural University (permit number: DK996).

**DNA extraction and metagenome sequencing**

Total genomic DNA was extracted from each rumen fluid sample with the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Germany). DNA concentration was measured using the UV-Vis NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and DNA integrity was checked using 1% agarose gel electrophoresis. Afterwards, metagenomic DNA libraries were constructed with an insert size of 350 bp for each sample with the TruSeq DNA PCR-free sample preparation kit (Illumina, San Diego, CA, USA). Metagenome sequencing was performed on an Illumina HiSeq-PE150 (150 bp paired-end sequencing).

**Metagenomic data processing**

**Quality control and metagenome assembly**

The raw data from Illumina HiSeq sequencing platform were preprocessed using Readfq (https://github.com/cjfields/readfq) to obtain clean data for subsequent analysis. The reads which contain low quality bases less than length of 40 bp, the N base that reached 10 bp and overlap above length of 15 bp were removed. In order to avoid the possibility of host pollution, the clean data was compared against the bovine reference genome UMD3.1.69 to filter out the reads that may be from the host with the parameters [27, 28] as follows: –end-to-end, –sensitive, -l 200 and -X 400. The filtered reads were assembled using SOAPdenovo 2.04 software (http://soap.genomics.org.cn/soapdenovo). The scaffolds longer than 500 bp were extracted for subsequently genetic prediction and annotation [29-31].

**Gene prediction and taxonomy prediction**

Using MetaGeneMark 2.10 software, open reading frame (ORF) prediction in the Scaftigs (>=500bp) was performed for each sample [32-34], and CD-HIT software was used for de-redundancy to obtain a non-redundant initial gene catalogue [35, 36]. DIAMOND[37] (V0.9.9, https://github.com/bbuchnk/diamond/) was used to blast the unigenes to the sequences of bacteria, fungi, archaea and viruses in the Non-Redundant Protein Sequence Database (NR) database in NCBI (https://www.ncbi.nlm.nih.gov/) for taxonomic analysis with the parameter setting as blastp -e 1e-5.

**Comparative analysis on microorganism abundance between high and low groups**
Krona analysis was performed to display relative abundance and abundance cluster heat map. Principal component analysis (PCA) [38] (R ade4 package, Version 2.15.3) and NMDS [39] (R vegan package, Version 2.15.3) decrease-dimension analysis were performed based on the abundance of each taxonomic hierarchy. Differences of microorganism abundance within groups and between groups were tested by Anosim analysis (R vegan package, Version 2.15.3). LEfSe and Metastats analysis were used to look for the species with different abundance between groups. LEfSe software was conducted with the default threshold LDA score of 3 [40]. For Metastats analysis, permutation test between groups was used for each taxonomy and get the raw P value, then Benjamini and Hochberg False Discovery Rate were used to correct P value and acquire q value [41].

Functional annotation analysis

DIAMOND software (V0.9.9) was adopted to blast the unigenes to functional databases to analyze the functions of rumen microorganisms, including Kyoto Encyclopedia of Gene and Genome (KEGG, http://www.kegg.jp/kegg/), Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG, http://eggnogdb.embl.de/#/app/home) and Carbohydrate-Active enzymes Database (CAZy, http://www.cazy.org/). The best Blast Hit was used for subsequent analysis. LEfSe and Metastats analysis were used to look for the different functions between the high and low groups.

Results

Sequencing of the rumen microbiota

With Illumina HiSeq PE-150 platform, we sequenced the microbiota DNA of each rumen fluid sample from the six lactating Holstein cows with extremely high and low milk PP and FP with three individuals in each group (high and low groups), and 76 Gbp clean data in total were generated after quality control (Supplementary Table 1). Through prediction and de-redundancy with MetaGeneMark and CD-HIT softwares, we identified a total of 1,078,009 ORFs with a total length of 667.24 Mbp in the scaffolds longer than 500 bp, from which 278,274 complete genes were annotated, accounting for 25.81% (Supplementary Table 2).

Taxonomic composition of rumen microbiota

With DIAMOND software, the BLAST search of the complete unigenes against the NR database at NCBI was conducted, as a result, the annotated phylum and genus accounted for 80.48% and 66.82%, respectively. Of these, 6977 kinds of microorganism species were detected, including 6259 bacteria, 397 eukaryota, 140 archaea, and 181 viruses. Taxonomic compositions of the rumen samples from six cows were displayed at phylum, class, order, family, genus and species levels (Supplementary Fig. 1). At the phylum level, as shown in Supplementary Fig. 2, the dominant bacterial phyla included Bacteroidetes (51.4%), Firmicutes (8.72%), Proteobacteria (5.77%) and Fibrobacteres (3.08%); at the genus level, the most abundant bacterial genera were Prevotella (38.48%), Fibrobacter (3.08%) and Bacteroides (2.47%);
and the dominant bacterial species included *Prevotella ruminicola* (3.85%), *Prevotella* sp.Ne3005 (3.33%), *Prevotella* sp.tc2-28 (2.77%), and *Prevotella* sp.tf2-5 (2.31%).

**Differential abundance of the rumen microbiome between high and low groups with PP and FP**

With PCA analysis, we observed that the six cows with extremely high and low milk PP and FP were obviously separated into two clusters based on the rumen microbiome abundance at genus and species levels ([Fig. 1A-B](#)), in which principal coordinate 1 accounted for 55.31%, 60.68% and principal coordinate 2 accounted for 14.21%, 12.81% of the total variation in two groups, respectively. Differential abundance comparison analysis showed there were significant differences on the abundances of rumen microorganisms between the cows in high and low groups (R = 0.889, P = 0.1; R = 1, P = 0.1; [Fig. 2](#)). Among the top ten abundant genera, *Prevotella* was the most genus with proportion of 42.8% and 34.2% in high and low groups, respectively; *Fibrobacter* accounted for 3.13% and 2.69%, and *Bacteroides* accounted for 3.02% and 2.25% in two groups, respectively; the abundances of another seven genera were 0.44% ~ 0.8% ([Supplementary Fig. 2E](#)). To identify differentially abundant species, we performed LEfSe analysis and detected 40 kinds of microbiota that had significantly differential abundances in the rumen of cows between high and low groups (LDA > 3, p < 0.05, [Fig. 3 and Fig. 4](#)), including 8 *Prevotella* sp., 2 unclassified _Bacteroidales_, 2 unclassified _Bacteria_ and 1 eukaryota of *Neocallimastix californiae* exhibited significantly higher abundances in high group, while 27 species showed significant enrichment in low group. Simultaneously, through Metastats analysis, 2797 microorganisms showed significantly differential abundances between two groups (p = 1.48E-05 ~ 0.049; q = 0.037 ~ 0.048), in which 38 species were commonly identified with both methods ([Supplementary Table 4](#)). Out of these, the top 35 abundant microorganisms(abundance >0.02%; [Fig. 5](#)) included 16 species from phylum *Bacteroidetes* in high group and 19 bacteria from phylum *Bacteroidetes, Firmicutes, Fibrobacteres* and *Proteobacteria* in low group; of note, 11 of 16 species in high group were *Prevotella* which functions as carbohydrate degradation.

**Functional enrichment of the rumen microbiome with differential abundances between high and low groups with PP and FP**

To understand the potential correlation of rumen microbiome with milk protein and fat traits, the functions of the differentially abundant rumen microbiome were determined by the Kyoto Encyclopedia of Gene and Genome (KEGG), Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) and Carbohydrate-Active enzymes Database (CAZy) functional annotation. Regarding KEGG, 108 pathways were significantly enriched in third-level pathways (LDA > 2.0 , P < 0.05, [Fig. 6 and Supplementary Table 5](#)), including 26 involved for the species with significantly high abundance in high group such as five “Metabolism”, six “Cellular Processes”, six “Environmental Information Processing”, seven “Organismal Systems”, and two “Human Diseases” pathways, while 42 pathways were enriched in low group. At the second level, 28 categories were observed, in which the most abundant pathways included “Glycan biosynthesis and metabolism”, “Carbohydrate metabolism”, “Amino acid metabolism”, “Metabolism of cofactors and vitamins” and “Signal transduction”. For KEGG
orthologues, 42 significant KOs were determined (LDA >2, \( P < 0.05 \), Fig. 7 and Supplementary Table 6) including 6 KOs (K01190, K05349, K01811, K03737, K01006 and K00688) related to carbohydrate, energy and pyruvate metabolisms were enriched in the rumen of cows in high group, and another 17 KOs with higher abundances in low group such as DNA-damage-inducible protein J (K07473), ATP-dependent DNA helicase RecG (K03655), and flagellin (K02406).

Based on the eggNOG database, seven significantly enriched pathways for the rumen microorganisms in high group were involved in carbohydrate transport and metabolism, signal transduction metabolism, energy production and conversion, translation ribosomal structure and biogenesis, lipid transport and metabolism, secondary metabolites biosynthesis and transport and catabolism, and posttranslational modification; four significantly enriched pathways in the low group included replication recombination and repair, amino acid transport and metabolism, coenzyme transport and metabolism, and cell motility (LDA >3, \( P < 0.05 \), Fig. 8 and Supplementary Table 7).

Based on the CAZyme database, a total of 215 genes encoding CAZymes were identified (Supplementary Table 8), including 96 glycoside hydrolases (GHs), 46 carbohydrate-binding modules (CBMs), 45 glycosyltransferase (GTs), 12 carbohydrate esterases (CEs), 11 polysaccharide lyases (PLs), and four auxiliary activities (AAs). Among these, GH51, GH97, GH31, GH2, GH3 and GH43 family glycoside hydrolases, and CE1 were significantly enriched in high group that were involved in deconstructing carbohydrates such as cellulose, hemicellulose and starch (LDA > 3, \( P < 0.05 \), Fig. 9 and Supplementary Table 9) indicating higher digestive ability for feedings of the rumen of cows in high group. In summary, \textit{Prevotella} bacteria was found significantly higher enriched in high group, which was capable of digesting fibrous hemicellulose starch, produced lactose and galactose, and then converted them to pyruvate. Pyruvate converted into VFA and amino acid, then transported through the blood to the mammary epithelium to make milk fat and protein (Fig. 10).

**Discussion**

In this study, we investigated the rumen microbiome from the milking Holstein cows in mid-lactation with extremely high and low milk protein percentage and fat percentages using metagenomics technology, and identified differential abundances of the microorganisms at phylum, genus and species levels. It was found that significantly differential species were significantly associated with milk protein and fat.

Here, we detected 6977 microorganisms in the rumen of cows, the top three most abundant phylum out of them were \textit{Bacteroidetes}, \textit{Firmicutes} and \textit{Proteobacteria}, which was consistent with the discovers in previous studies [42-44]. In addition, we found the predominant anaerobic fungi was \textit{Neocallimastigomycota} that was consistent with the result reported by Zhang et al. [43]. However, Huang et al. found \textit{Actinobacteria} was one of the most abundant predominant phylum besides \textit{Bacteroidetes} and \textit{Firmicutes} in the rumen of lactating Chinese Holstein cows with 16S rRNA sequencing[45]. This was likely due to the different feeding management and environment of the farms where cows were collected from that in this study. At the genera level, we identified that \textit{Prevotella} was the most abundant genus in
the rumen of lactating cows with high protein trait. This is coincident with the previous report by Xue et al. [44].

We observed 2798 microbial species that showed significantly differential abundances in the rumen fluid between the lactating cows with extremely high and low milk protein percentage and fat percentages. Based on KEGG, eggNOG and CAZy databases, it was found that such species were mainly involved in amino acid, carbohydrate, pyruvate and lipid metabolism and transportation. Especially, majority of the most abundant species in high group belonged to the *Prevotella* genus. It was well known that *Prevotella* genus could metabolize cellulose and starch, its fermentation products include acetate, butyrate and propionate[46]. The previous studies indicated that higher *Prevotella* abundance in the rumen accelerated the decomposition of fructose and starch[47] and could produce propionate or succinate and acetate[48]. Besides, some studies found that *Prevotella* was also polysaccharide-degrading genus, had the ability to degrade mucin and plant carbohydrates [49, 50]. Previous research had shown that the P. bryantii 25A in *Prevotella* could increase ruminal fermentation products and milk fat concentration after supplied into early lactating cows[51]. Our results were consistent with these reports. Thus, since rumen fluid contained more *Prevotella* species in the high group, we speculated it could digest the cellulose, hemicellulose and starch well in the feed and degrade them into galactosidase and glucose. Under the action of microorganisms in the rumen, galactosidase and glucose converted to pyruvate. Then, part of pyruvate were broken down into VFA, which provided a sufficient material basis for lactation in cows. The rumen VFA in dairy cows were basically acetate, propionate and butyrate. After passing through the rumen wall, acetate was transported to the liver via the blood[52]. Butyrate was conversion to β-hydroxybutyric acid (BHBA) and absorbed by the liver. In the liver, these substances were converted into cholesterol. Finally, cholesterol was transported to the mammary gland in the form of lipoproteins, involved in the synthesis of milk fat[53-55]. Propionate was the main precursor of glucose synthesis in ruminants, which was beneficial to the supply and transformation of energy in the body and provided energy for protein synthesis. The increase of propionate could also stimulate the secretion of insulin, the blood flow of the breast, to promote the synthesis of milk protein [56-58]. Amino acids produced in the rumen were used by the small intestine and converted to FAA. FAA was absorbed as it flowed through the mammary gland to synthesize milk protein. Additionally, *Neocallimastix californiae*, a kind of anaerobic fungi, was highly enriched in our high group which had been reported that it could reduce the inner tension of plant fiber and make it easier to be degraded by rumen microbiomes[59]. In subsequent experiments, we made bacteria with significant differences into enzyme preparations and fed them to cows to test whether the bacteria could affect milking traits. This might warrant further studies in the future.

Interestingly, in top 35 abundant microorganisms, 16 species with high abundance in the high group belong to Bacteroidetes genera, and 11 of 16 species in high group were *Prevotella* which functions as carbohydrate degradation. While 19 species with high abundance in the low group were distributed in four genera. The high group, with high milk fat and protein, had the characteristics of lower diversity and higher advantage in genus content. As we all known, microbial diversity had an important effect on cow metabolism. And Shabat et al.’ study found that lower richness of microbiome gene content and taxa was
tightly linked to higher feed efficiency [60]. The diversity and abundance of microbiomes, and mutualism between microbiomes and anaerobic bacteria were significantly associated with milking traits in dairy cow.

**Conclusion**

The present study obtained the rumen microbiome profile of lactating Holstein cows using microgenomic sequencing, and identified 38 top abundant species that showed significantly differential richness between the cows with extremely high and low milk PP and FP. Function annotation indicated that the differential species with higher abundance in high group were associated with carbohydrate, amino acid, pyruvate, insulin and lipid metabolism and transportation, implying their higher capability of digesting feed and subsequently providing substrate for milk composition synthesis in mammary gland.

**Abbreviations**

PP: protein percentage  
FP: fat percentage  
VFA: volatile fatty acids  
FAAs: free amino acids  
ORF: open reading frame  
NR: Non-Redundant Protein Sequence Database  
PCA: principal component analysis  
KEGG: Kyoto Encyclopedia of Gene and Genome  
eggNOG: Non-supervised Orthologous Groups  
CAZy: Carbohydrate-Active enzymes Database  
GHs: glycoside hydrolases  
CBMs: carbohydrate-binding modules  
GTs: glycosyltransferase  
CEs: carbohydrate esterases  
PLs: polysaccharide lyases
AAs: auxiliary activities

BHBA: β-hydroxybutyric acid

**Declarations**

**Acknowledgements**

Not applicable

**Availability of data and materials**

The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Funding**

This work was financially supported by the National Natural Science Foundation of China (31872330, 31872330, 31802041), and the Program for Changjiang Scholar and Innovation Research Team in University (IRT_15R62).

**Authors’ contributions**

DS conceived and designed the experiments, XW prepared the DNA samples for metagenomic sequencing and analyzed the data, and the manuscript was prepared by XW and DS. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All experiments were carried out in accordance with Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at China Agricultural University (Beijing, China; permit number: DK996).

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Pereira PC. Milk nutritional composition and its role in human health. Nutrition. 2014 Jun;30:619-27.
2. Morgavi DP, Kelly WJ, Janssen PH, Attwood GT. Rumen microbial (meta) genomics and its application to ruminant production. Animal. 2013 Mar;7 Suppl 1:184-201.

3. Eisler MC, Lee MR, Tarlton JF, Martin GB, Beddington J, Dungait JA, et al. Agriculture: Steps to sustainable livestock. Nature. 2014 Mar 6;507:32-4.

4. Seshadri R, Leahy SC, Attwood GT, Teh KH, Lambie SC, Cookson AL, et al. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. Nat Biotechnol. 2018 Apr;36:359-367.

5. Russell JB, Rychlik JL. Factors that alter rumen microbial ecology. Science. 2001 May 11;292:1119-1122.

6. Herrero M, Havlik P, Valin H, Notenbaert A, Rufino MC, Thornton PK, et al. Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. Proc Natl Acad Sci U S A. 2013 Dec 24;110:20888-93.

7. Baldwin RLt, Connor EE. Rumen Function and Development. Vet Clin North Am Food Anim Pract. 2017 Nov;33:427-439.

8. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, et al. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem. 2003 Mar 28;278:11312-9.

9. Ahmed K, Tunaru S, Offermanns S. GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors. Trends Pharmacol Sci. 2009 Nov;30:557-62.

10. Kondo T, Kishi M, Fushimi T, Kaga T. Acetic acid upregulates the expression of genes for fatty acid oxidation enzymes in liver to suppress body fat accumulation. J Agric Food Chem. 2009 Jul 8;57:5982-6.

11. Taggart AK, Kero J, Gan X, Cai TQ, Cheng K, Ippolito M, et al. (D)-beta-Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. J Biol Chem. 2005 Jul 22;280:26649-52.

12. Xu C, Wang Z, Zhang RH, Zhang HY, Fu SX, Xia C. Effect of NEFA and glucose levels on CPT-I mRNA expression and translation in cultured bovine hepatocytes. J Vet Med Sci. 2011 Jan;73:97-101.

13. Glatz JFC, Luiken JJFP, Bonen A. Membrane Fatty Acid Transporters as Regulators of Lipid Metabolism: Implications for Metabolic Disease. Physiological Reviews. 2010 Jan;90:367-417.

14. Smith JL, Lear SR, Forte TM, Ko W, Massimi M, Erickson SK. Effect of pregnancy and lactation on lipoprotein and cholesterol metabolism in the rat. J Lipid Res. 1998 Nov;39:2237-49.

15. Chanishvili N. Phage Therapy-History from Twort and d'Herelle Through Soviet Experience to Current Approaches. Adv Virus Res. 2012;83:3-40.
16. Handelsman J. Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev. 2004 Dec;68:669-85.

17. Faust K, Sathirapongsasuti JF, Izard J, Segata N, Gevers D, Raes J, et al. Microbial co-occurrence relationships in the human microbiome. PLoS Comput Biol. 2012;8:e1002606.

18. Brown MV, Lauro FM, DeMaere MZ, Muir L, Wilkins D, Thomas T, et al. Global biogeography of SAR11 marine bacteria. Mol Syst Biol. 2012 Jul 17;8:595.

19. Pasolli E, De Filippis F, Mauriello IE, Cumbo F, Walsh AM, Leech J, et al. Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. Nat Commun. 2020 May 25;11:2610.

20. Bisanz JE, Reid G. Unraveling How Probiotic Yogurt Works. Sci Transl Med. 2011 Oct 26;3.

21. Huang K, Mao Y, Zhao F, Zhang XX, Ju F, Ye L, et al. Free-living bacteria and potential bacterial pathogens in sewage treatment plants. Appl Microbiol Biotechnol. 2018 Mar;102:2455-2464.

22. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol. 2013 Jul;14:685-90.

23. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012 Sep 13;489:242-249.

24. Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, et al. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science. 2011 Jan 28;331:463-7.

25. Jewell KA, McCormick CA, Odt CL, Weimer PJ, Suen G. Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. Appl Environ Microb. 2015 Jul;81:4697-4710.

26. Jami E, White BA, Mizrahi I. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PLoS One. 2014;9:e85423.

27. Karlsson FH, Tremaroli V, Nookaew I, Bergstrom G, Behre CJ, Fagerberg B, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013 Jun 6;498:99-103.

28. Karlsson FH, Fak F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, et al. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat Commun. 2012 Dec;3.

29. Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome in liver cirrhosis. Nature. 2014 Sep 4;513:59-64.

30. Mende DR, Waller AS, Sunagawa S, Jarvelin AI, Chan MM, Arumugam M, et al. Assessment of metagenomic assembly using simulated next generation sequencing data. PLoS One. 2012;7:e31386.
31. Nielsen HB, Almeida M, Juncker AS, Rasmussen S, Li JH, Sunagawa S, et al. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat Biotechnol. 2014 Aug;32:822-828.

32. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010 Mar 4;464:59-65.

33. Zeller G, Tap J, Voigt AY, Sunagawa S, Kultima JR, Costea PI, et al. Potential of fecal microbiota for early-stage detection of colorectal cancer. Mol Syst Biol. 2014 Nov 28;10:766.

34. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et al. An integrated catalog of reference genes in the human gut microbiome. Nat Biotechnol. 2014 Aug;32:834-41.

35. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006 Jul 1;22:1658-9.

36. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics. 2012 Dec 1;28:3150-2.

37. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015 Jan;12:59-60.

38. Avershina E, Frisli T, Rudi K. De novo semi-alignment of 16S rRNA gene sequences for deep phylogenetic characterization of next generation sequencing data. Microbes Environ. 2013;28:211-6.

39. Noval Rivas M, Burton OT, Wise P, Zhang YQ, Hobson SA, Garcia Lloret M, et al. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. J Allergy Clin Immunol. 2013 Jan;131:201-12.

40. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011 Jun 24;12:R60.

41. White JR, Nagarajan N, Pop M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput Biol. 2009 Apr;5:e1000352.

42. Pitta DW, Indugu N, Kumar S, Vecchiarelli B, Sinha R, Baker LD, et al. Metagenomic assessment of the functional potential of the rumen microbiome in Holstein dairy cows. Anaerobe. 2016 Apr;38:50-60.

43. Zhang J, Shi H, Wang Y, Li S, Cao Z, Ji S, et al. Effect of Dietary Forage to Concentrate Ratios on Dynamic Profile Changes and Interactions of Ruminal Microbiota and Metabolites in Holstein Heifers. Front Microbiol. 2017;8:2206.

44. Xue MY, Sun HZ, Wu XH, Liu JX, Guan LL. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance.
45. Huang S, Ji S, Yan H, Hao Y, Zhang J, Wang Y, et al. The day-to-day stability of the ruminal and fecal microbiota in lactating dairy cows. Microbiologyopen. 2020 May;9:e990.

46. Shah HN, Collins DM. Prevotella, a new genus to include Bacteroides melaninogenicus and related species formerly classified in the genus Bacteroides. Int J Syst Bacteriol. 1990 Apr;40:205-8.

47. Metzler-Zebeli BU, Schmitz-Esser S, Klevenhusen F, Podstatzky-Lichtenstein L, Wagner M, Zebeli Q. Grain-rich diets differently alter ruminal and colonic abundance of microbial populations and lipopolysaccharide in goats. Anaerobe. 2013 Apr;20:65-73.

48. Strobel HJ. Vitamin B12-dependent propionate production by the ruminal bacterium Prevotella ruminicola 23. Appl Environ Microbiol. 1992 Jul;58:2331-3.

49. Lamendella R, Domingo JW, Ghosh S, Martinson J, Oerther DB. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiol. 2011 May 15;11:103.

50. Patel DD, Patel AK, Parmar NR, Shah TM, Patel JB, Pandya PR, et al. Microbial and Carbohydrate Active Enzyme profile of buffalo rumen metagenome and their alteration in response to variation in the diet. Gene. 2014 Jul 15;545:88-94.

51. Chiquette J, Allison MJ, Rasmussen MA. Prevotella bryantii 25A used as a probiotic in early-lactation dairy cows: Effect on ruminal fermentation characteristics, milk production, and milk composition. Journal of Dairy Science. 2008 Sep;91:3536-3543.

52. Vazirigohar M, Dehghan-Banadaky M, Rezayazdi K, Nejati-Javaremi A, Mirzaei-Alamouti H, Patra AK. Short communication: Effects of diets containing supplemental fats on ruminal fermentation and milk odd- and branched-chain fatty acids in dairy cows. J Dairy Sci. 2018 Jul;101:6133-6141.

53. Castaneda-Gutierrez E, Pelton SH, Gilbert RO, Butler WR. Effect of peripartum dietary energy supplementation of dairy cows on metabolites, liver function and reproductive variables. Anim Reprod Sci. 2009 Jun;112:301-15.

54. Drackley JK. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci. 1999 Nov;82:2259-73.

55. Mach N, Zom RL, Widjaja HC, van Wikselaar PG, Weurding RE, Goselink RM, et al. Dietary effects of linseed on fatty acid composition of milk and on liver, adipose and mammary gland metabolism of periparturient dairy cows. J Anim Physiol Anim Nutr (Berl). 2013 May;97 Suppl 1:89-104.

56. Raggio G, Lemosquet S, Lobley GE, Rulquin H, Lapierre H. Effect of casein and propionate supply on mammary protein metabolism in lactating dairy cows. J Dairy Sci. 2006 Nov;89:4340-51.
57. Liu Q, Wang C, Guo G, Huo WJ, Zhang SL, Pei CX, et al. Effects of branched-chain volatile fatty acids on lactation performance and mRNA expression of genes related to fatty acid synthesis in mammary gland of dairy cows. Animal. 2018 Oct;12:2071-2079.

58. Oba M, Allen MS. Dose-response effects of intrauminal infusion of propionate on feeding behavior of lactating cows in early or midlactation. J Dairy Sci. 2003 Sep;86:2922-31.

59. Chang J, Park H. Nucleotide and protein researches on anaerobic fungi during four decades. J Anim Sci Technol. 2020 Mar;62:121-140.

60. Shabat SK, Sasson G, Doron-Faigenboim A, Durman T, Yaacoby S, Berg Miller ME, et al. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 2016 Dec;10:2958-2972.

Figures

**Figure 1**

Microbial composition at the genus and species levels differs between rumen of high and low milk PP and FP. (A) Principal component analysis (PCA) of metagenomics based genera. (B) PCA of metagenomics based species.
Figure 1

Microbial composition at the genus and species levels differs between rumen of high and low milk PP and FP. (A) Principal component analysis (PCA) of metagenomics based genera. (B) PCA of metagenomics based species.
Figure 2

Anosim analysis based on genus and species levels. (A) Anosim analysis based on genus level. (B) Anosim analysis based on species level. The R-value is between (-1,1) and the R-value is greater than 0, indicating that the difference between the groups is significant.
Figure 2

Anosim analysis based on genus and species levels. (A) Anosim analysis based on genus level. (B) Anosim analysis based on species level. The R-value is between (-1,1) and the R-value is greater than 0, indicating that the difference between the groups is significant.
Figure 3

LDA Effect Size (LEfSe) analysis of rumen microbiota between two groups. Note: LDA (linear discriminant analysis) plot indicates biomarkers found by ranking accordingly to their effect size (4.0) of the species.
**Figure 3**

LDA Effect Size (LEfSe) analysis of rumen microbiota between two groups. Note: LDA (linear discriminant analysis) plot indicates biomarkers found by ranking accordingly to their effect size (4.0) of the species.
Figure 4

Percent composition and significance of species in the rumen fluid.
Figure 4

Percent composition and significance of species in the rumen fluid.
| Group                                      | Phylum             |
|--------------------------------------------|--------------------|
| Bacteroidales bacterium WCE2008            | Bacteroidetes      |
| Bacteroidales bacterium WCE2004            |                    |
| bacterium P201                             | Firmicutes         |
| bacterium F083                             |                    |
| Prevotella sp. tc2–28                      | Unclassified       |
| Prevotella sp. P6B4                        |                    |
| Prevotella sp. ne3005                      |                    |
| Prevotella sp. MA2016                      |                    |
| Prevotellaceae bacterium MN60              | Proteobacteria     |
| Prevotellaceae bacterium HUN156            |                    |
| Prevotella sp. BP1–148                     |                    |
| Prevotella sp. BP1–145                     |                    |
| Prevotella ruminicola                      |                    |
| Prevotella brevis                          |                    |
| Prevotella sp. tf2–5                       |                    |
| Bacteroides sp. 43_108                     |                    |
| Prevotella bryantii                        |                    |
| Prevotella albensis                        |                    |
| Selenomonas ruminantium                    |                    |
| Succinivibrionaceae bacterium XPB1003      |                    |
| Succinobrevibacter sp. UWB2                |                    |
| Succinobrevibacter sp. UWR2                |                    |
| Succinobrevibacter sp. UWH4                |                    |
| Ruminococcus flavifaciens                  |                    |
| Lachnospiraceae bacterium XPB1003          |                    |
| Schwartzia succinivorans                  |                    |
| Fibrobacter sp. CAG:777                    |                    |
| Succinichelinium ruminis                   |                    |
| Fibrobacter sp. UWB2                       |                    |
| Fibrobacter sp. UWR2                       |                    |
| Succiniclasticum ruminis                   |                    |
| Fibrobacter sp. UWH4                       |                    |
| Ruminococcus flavifaciens                  |                    |
| Lachnospiraceae bacterium XPB1003          |                    |
Figure 5

Species level relative abundance clustering heat map
Figure 5

Species level relative abundance clustering heat map
Figure 6

LDA Effect Size (LEfSe) analysis the function of KEGG between two groups.
Figure 6

LDA Effect Size (LEfSe) analysis the function of KEGG between two groups.
Figure 7

LDA Effect Size (LEfSe) analysis the function of KOs between two groups.
Figure 7

LDA Effect Size (LEfSe) analysis the function of KOs between two groups.
Figure 8

LDA Effect Size (LEfSe) analysis the function of eggNOG between two groups.

Figure 8

LDA Effect Size (LEfSe) analysis the function of eggNOG between two groups.
Figure 9

LDA Effect Size (LEfSe) analysis the function of CAZy between two groups.
Figure 9

LDA Effect Size (LEfSe) analysis the function of CAZy between two groups.
Figure 10

Consolidation of results from the KEGG, CAZy, eggNOG database analyses. Note: Green: enriched in microbiomes, enzyme and pathway. Blue: decomposition products. Yellow: plant biomass. Ko00052: galactose metabolism, Ko00511: glycan degradation.
Figure 10

Consolidation of results from the KEGG, CAZy, eggNOG database analyses. Note: Green: enriched in microbiomes, enzyme and pathway. Blue: decomposition products. Yellow: plant biomass. Ko00052: galactose metabolism, Ko00511: glycan degradation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure.docx
- SupplementaryFigure.docx
- SupplementaryTable.xlsx
- SupplementaryTable.xlsx