Characterization of bacterial community of rumen from bovine during laminitis challenge by high-throughput sequencing

Jian Guo

Jilin University

Haowen Sun

Jilin University

T. Maimai

Jilin University

Caijun Zhao

Jilin University

Yongguo Cao

Jilin University

Naisheng Zhang

Jilin University

Xiaoyu Hu

Jilin University

Yunhe Fu (✉ fuyunhesky@163.com)

Jilin University <https://orcid.org/0000-0002-3258-2610>

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Abstract

Background: Laminitis is a classic problem in the dairy industry, which can cause a great economic loss. However, the etiology and pathogenesis of laminitis have yet to be understood. In recent years, the microbiota has been the focus of much investigation in the search for various diseases. The present study aimed to explore the relationship between ruminal bacterial microbiota and laminitis.

Results: The serum of healthy and laminitis bovines (n=8, respectively) collected from farms was used to detect concentrations of LPS, lactic acid, and histamine by the detection kits. This study used 16S rRNA sequencing to identify the differences in the bacterial community. The results showed that there was a significant increase in LPS and lactic acid in the laminitis group. Furthermore, microbial data analysis revealed that the laminitis group increased the abundance of bacteria with acid metabolites, such as *Candidatus Saccharimonas*, *Saccharofermentans*, *Erysipelotrichaceae* UCG-009, and *Erysipelotrichaceae* UCG-008, and [*Clostridium*] *papyrosolvens* and *Ruminococcaceae* bacterium AE2021.

Conclusions: In summary, the changes in ruminal bacteria may potentially serve as the risk of laminitis.

Background

The growing productivity of bovine has been increasing the risk of metabolic diseases such as foot disease[1]. Laminitis is one of the major reasons for enormous economic losses in the bovine dairy industry[2] and make up 62% of lameness cases[3]. Succinctly stated, laminitis is an aseptic inflammation of the sensitive lamina of the foot[4]. Laminitis can be categorized according to the severity and duration such as acute, subacute, chronic and subclinical[5]. In the acute and subacute stage of the disease, the cows' manifest lameness in all limbs[2], however, in chronic form, there is no obvious systemic symptoms and only the claw show changes. The hoof is elongated with a flattened and broadened, named "slipper foot"[6-8]. The subclinical laminitis has been discovered by 1979[9]. Changes in posture and locomotion are inapparent and occur after sole ulcers and white-line disease by two to three months[9, 10]. In spite of its importance, the physiopathology of laminitis remains to be elucidated[11]. This happens as the result of various factors related to breeding conditions and metabolic insult, especially in animal nutrition. The commonly admissible factors are toxic substances, such as histamine, lactic acid and lipopolysaccharide (LPS)[12], which would cause vascular lesions and degradation of the suspensory apparatus of the third phalanx within the digit[3, 13, 14].

In bovine, host-rumen-microbe interactions play a critical role in the maintenance of physiological activity and therefore could be a contributing factor to affect health[15]. It is now well established that the disturbance of ruminal microbiota can induce bovine laminitis, and ruminal factors found to be influencing laminitis have been explored in several studies. The main reason is the indigestions which were caused by ruminal acidosis[16]. To improve the milk yield, the concentrates are overused, which can destroy the balance of the ruminal flora. Then harmful metabolites of rumen bacteria such as lipopolysaccharide (LPS), histamine and lactic acid may increase[16-18]. Recently investigators have

examined the effects of LPS on inflammatory reactions[19], which are the main pathological changes incipient stage of laminitis[20]. Besides, previous research has shown that intradermal injection of LPS into bovine can induce laminitis[21]. In ruminal acidosis, vasoactive substances (LPS and histamine) release and lead to vasoconstriction and dilation, which will destroy the microvasculature of the corium[22, 23]. The research to date has been able to show a link between laminitis and lactic acidosis following carbohydrate overload[3]. Such approaches, however, have failed to address the relation of the ruminal microbiota to laminitis. This paper aims to provide empirical and theoretical evidence for the claim that the changes in characterization of the bacterial community will induce bovine laminitis.

Results

LPS, lactic acid, and histamine in serum

As shown in Fig. 1A-C, the concentration of LPS and lactic acid in the laminitis group obviously increase, while that of histamine in serum remained similar.

The composition of the ruminal bacterial community

PCR has been utilized to test for The V4 region of the ruminal bacterial 16S ribosomal RNA (rRNA) gene, to identify the characterization of ruminal bacterial community from nine ruminal fluid samples (8 from control and 8 from laminitis). Rarefaction curves displayed the majority of bacterial diversity had been proved by the sampling depth had sufficient sequences (see supplementary Fig. S1). The difference between control and laminitis group in both community richness and diversity were compared using observed species, Chao 1, and ace and Shannon index and Simpson index. There was no significant difference. Interestingly, the nonmetric multidimensional scaling (NMDS) ordination was observed that the bacterial community of samples of healthy bovines was separated from that of laminitis samples using the Bray-Curtis dissimilarity (Fig. 2), the value of stress was 0.104.

Changes of ruminal bacterial community at the phylum level

At the phyla level, these bacterial sequences obtained from all the bovine comprised 21 phyla and shared the same in both groups (see supplementary Table 1). Among them, Bacteroidetes (control vs laminitis, 50.64% vs 42.28%) and Firmicutes (control vs laminitis, 34.85% vs 43.64%) were the most abundant phyla in the bovine ruminal bacterial community. In addition, Proteobacteria (control vs laminitis, 8.03% vs 2.66%), Spirochaetes (control vs laminitis 1.08% vs 0.48%), Tenericutes (control vs laminitis 1.29% vs 3.65%), Euryarchaeota (control vs laminitis 0.86% vs 1.66%), and Cyanobacteria (control vs laminitis 0.97% vs 0.95%) were detected and the relative abundance at or near 1% or more in the bovine ruminal microbiota (As shown in Fig. 3A and Supplementary Table 1). The purpose of T-test was used to compare the difference between the bacterial abundant phyla between healthy and laminitis. The results showed that the relative abundance of Tenericutes, Saccharibacteria, and SR1 (Absconditabacteria) significantly increased. (As shown in Fig. 3B)

DIChanges of ruminal bacterial community at the genus and species levels

At the genus level, these bacterial sequences detected from all the animals comprised 245 genera. The results obtained from the preliminary analysis of the dominant genus are displayed in Fig. 4A and supplementary table 2; *Prevotella* 1 (control vs laminitis, 20.44% vs 13.46%), *Succiniclasicum* (control vs laminitis, 7.77% vs 7.06%), *Succinivibrionaceae* UCG-002 (control vs laminitis, 4.34% vs 0.79%), *Christensenellaceae* R-7-group (control vs laminitis, 4.63% vs 7.94%), *Succinivibrionaceae* UCG-001 (control vs laminitis 2.20% vs 0.30%), *Ruminococcaceae* NK4A214-group (control vs laminitis, 3.52% vs 5.11%), *Ruminococcaceae* UCG-014 (control vs laminitis, 2.12% vs 3.29%) and *Rikenellaceae* mRC9 gut group (control vs laminitis, 3.85% vs 4.24%). T-test showed that the relative abundance of *Candidatus* *Saccharimonas*, *Saccharofermentans*, and *Erysipelotrichaceae* UCG-009 were remarkably increased. Meanwhile, none of the differences of the other genus were statistically significant (Fig. 4B).

At the species level, the T-test analyzed presented that species of [*Clostridium*] *papyrosolvens* and *Ruminococcaceae* bacterium AE2021 were significantly increased. while, the relative abundance of *Prevotella ruminicola*, *Prevotella* sp. CA17 and *Treponema saccharophilum* were decreased in ruminal microbiota from laminitis samples (Fig. 5A-E). Furthermore, the core genera shared by the healthy and laminitis bovine were used to evaluate the link between unique bacterial microbiota and laminitis. The Venn diagram illustrated that the proportion of the main genera of the healthy group was nearly 90.66%, that of the laminitis group was responsible for 92.74% (Fig. 5F). These results may explain the relatively good correlation between changes in bacterial abundance and interactions among shared bacteria and bovine laminitis. The biomarker analysis by linear discriminant analysis (LDA) effect size (LEfSe) and a cladogram generated from LEfSe analysis on the microbiota community of rumen to prove that theory. At the genus levels, the obvious biomarkers were *Ruminococcaceae* UCG 014, *Candidatus* *Saccharimonas*, *Saccharofermentans* and *Succinivibrionaceae* UCG 002. While that of species levels was no significant biomarkers (Fig. 6A-B). Interestingly, the bacteria that degrade cellulose and produce volatile fatty acids (VFA) was observed to influence the bovine laminitis.

Discussion

Bovine laminitis, one of the most cost of lameness conditions, is an economic drain on producers and is a welfare issue. it is generally accepted that micro-circulation of blood disorder within the corium is the main pathogenesis of laminitis. The local blood circulatory disorder is related to destroy the dermal-epidermal junction between the claw wall, and the bone (otherwise known as the third phalanx (P3) within the claw[24]. However, laminitis does not have one specific cause. There is a critical link between nutrition, acidosis, and laminitis[3]. Surveys such as that conducted by Raymond J. have shown that disorders can promote the cow to laminitis[25]. E.Nocek shows how, in the past, research into bovine acidosis was mainly concerned with laminitis[3]. During the acidosis, vasoactive substances (lactic acid and endotoxins) are released and induce micro-circulation of blood disorder, which leads to the corium tissue damage and causes laminitis[23].

Endotoxins, lipopolysaccharides (LPS), is a major component of the outer membrane of gram-negative bacteria. As the main vasoactive substance, LPS plays a key role in inflammatory reactions. When it happens ruminal acidosis, plenty of gram-negative bacteria are dead and LPS release. In the early stages of laminitis, the inflammatory reactions play a key role. LPS is absorbed into the blood through the ruminal wall, and then reach the micro-circulation of the claw by blood circulation. The local LPS induce several inflammatory effects, such as the activation of cytokines and acute-phase protein release, thrombocytopenia, leukopenia followed by leukocytosis[26]. These changes would induce the damage of the claw to cause laminitis[27]. Besides, recent evidence suggests that the laminitis pathology changes could be made by injection of LPS[21]. In this study, the concentration of LPS significantly increased in the laminitis group compared with the healthy group.

Histamine and lactic acid, as the same vasoactive substances as LPS, are associated with laminitis[28]. Both of them can present a risk to claw deterioration and the laminitis process, include vasoconstriction, dilation, edema and thrombosis, and interfere with the migration and function of defense cells such as neutrophils[28]. Previous studies have reported the increase of histamine and lactic acid in bovine serum during laminitis[3, 5, 28]. Our experiments confirm previous results, lactic acid of laminitis bovine serum went up. However, contrary to expectations, we did not find a significant difference in histamine between the healthy and laminitis groups.

There is evidence that the ruminal bacterial community plays a crucial role in the regulating pathologic of organism in developing disease[29]. The role of microbial populations has received increased attention across several disciplines in recent years. Therefore, a number of cross-sectional studies suggest an association between microbiota and its metabolite and laminitis. The proportion of phylum Firmicutes and genera *Streptococcus* and *Lactobacillus* significantly grew, while the abundance of phyla Bacteroidetes and Fibrobacteres and genera *Butyrivibrio* and *Ruminococcus* dramatically declined during the period of laminitis[30]. The capacity to produce vasoactive amines by *Streptococcus bovis* and 5 *Lactobacilli* spp are known to increase the risk of laminitis[31]. In the literature, *S. bovis* can increase other bacteria and produce lactic acid together, which is associated to bring down the pH[32]. In the present study, we have made the observation in significant differences in the bacterial community between the two groups using NMDS. In brief, the increased genus compared to the healthy group were *Candidatus Saccharimonas*, *Saccharofermentans*, *Erysipelotrichaceae* UCG-009, and *Erysipelotrichaceae* UCG-008, most of which were associated with fermentable diets and reduced ruminal pH[33-35]. With the reduction of pH, plenty of the gram-negative bacteria died and releases a number of LPS. At the species level, [*Clostridium*] *papyrosolvens* and *Ruminococcaceae* bacterium AE2021 were significantly increased. As argued by Boonsaen P, *Ruminococcaceae* (e.g. *Ruminococcus flavefaciens* and *Ruminococcus albus*) can produce lactate and propionate[36]. These results suggested that the changes of the bacterial community may a factor causing laminitis by influencing rumen metabolisms, such as the increase of LPS and lactate.

Conclusion

In conclusion, the study showed that the vasoactive substances associated with laminitis increased such as LPS and lactate. Besides, there were several differences between healthy and laminitis bovines in the bacterial community. At genus and species level, the quantity of *Candidatus Saccharimonas*, *Saccharofermentans*, *Erysipelotrichaceae* UCG-009, and *Erysipelotrichaceae* UCG-008, and [*Clostridium*] *papyrosolvens* and *Ruminococcaceae* bacterium AE2021 increased. Briefly, targeting ruminal microbiota may play a critical role in preventing bovine laminitis.

Methods

Farms and animals

A cross-sectional study was carried out at smallholder dairy farms located in Linqu Country, Weifang city, Shandong province, China, between November 2018 and June 2019. Five farms were selected by sampling from a list of farms that were willing to participate in our study. To begin this process, the animals were selected by lameness examination and divided into the health group and lameness group. The lameness bovines were then further examined by shoeing to identify laminitis[37]. Finally, the laminitis samples were extracted using the four-point scale for the laminitis scoring system[38]. The blood was collected with vacutainer tubes with EDTA and rumen fluid with sterile 50 mL centrifuge tubes by rumen puncture[39]. Then the samples were kept at 80 °C for microbiota analysis. After the samples were collected, the cows were normally raised and treated.

Lipopolysaccharide concentration detection

Blood was centrifuged at 14000 g for 30 min at 4 °C and then the supernatants were transferred into a sterile and depyrogenated glass tube. Then the serum was quantified using a chromogenic endpoint assay (Chinese Horseshoe Crab Reagent Manufactory Co.,Ltd., Xiamen, China) with a minimum detection limit of 0.01 EU/mL under the manufacturer's instructions.

Lactic acid and histamine concentrations detection

Serum was extracted from the blood by centrifuging at 14000 g for 30 min at 4 °C, and then was detected the concentration of lactic acid and histamine using the detection kits according to the manufacturer's instructions (Suzhou feiya Biological Technology, Suzhou, China).

DNA extraction, Illumina MiSeq sequencing, bioinformatics analyses

The genome DNA of ruminal fluid was extracted using a CTAB/SDS method. The DNA concentration and purity were detected by 1 % agarose gels. To amplify the 16S rRNA, barcoded primers (16S V4:515F-806R) targeting the V4 region was used. The PCR reactions were conducted with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed in equal ratios and then purified with a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). The library quality was evaluated by a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Finally, the library was

sequenced on an Illumina HiSeq 2500 platform, and 250 bp paired-end reads were generated. Bacterial community diversity and richness were analyzed by ACE, Chao 1, the Shannon index, the Simpson index, and the observed species. The distance of bacterial community between control and laminitis was evaluated by NMDS of Bray-Curtis dissimilarity. The bacterial taxa differentially between control and laminitis were evaluated by LEfse, and the Venn diagram was conducted to evaluate the numbers of core genera in the ruminal contents from the control and the laminitis group bovine.

Statistical analysis

Statistical analysis was arranged by GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA). All data are presented as means $\pm$ SEM. To compare the differences of the various experimental groups, a one-way ANOVA (Dunnett's t-test) and the two-tailed t-test were used. The $P < 0.05$ or $P < 0.01$ was considered Statistical significance.

Abbreviations

LPS: lipopolysaccharide; EDTA: ethylenediamine tetraacetic acid; NMDS: nonmetric multidimensional scaling; LDA: linear discriminant analysis; VFA: volatile fatty acids.

Declarations

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Author's contributions

Yunhe Fu and Xiaoyu Hu designed and performed the study, and drafted the manuscript. Jian Guo performed the rumen fluid and blood collection, and microbiota analysis, and contributed to writing the manuscript. Haowen, Sun performed the LPS, lactic acid and histamine analysis. T. Maimai and Caijun Zhao performed the literature review. Yongguo Cao and Naisheng Zhang performed the supervised the research project and reviewed the paper.

Ethics approval and consent to participate

This study was carried out following the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Jilin University, and we have a permission to collect animals samples from the farm owner.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Taxonomic biomarkers. A: Linear discriminative analysis (LDA) effect size LefSe analysis between the control (red) and laminitis (green). B: Cladogram showing differentially abundant taxonomic clades with an LDA score > 3.5 among laminitis and controls, $p < 0.05$.

Figure 2

At the species level, differences between laminitis and healthy rumen microbiota by the two-tailed t-test (A-E). The values presented are the mean ± SEM. *P < 0.05 and **P < 0.01 are significantly different from laminitis group. F: Mutual and particular operational taxonomic unit (OTU) number of rumen microbiota in laminitis and healthy samples.

Figure 3

Relative abundances of most abundant genus levels (near 1% or more in all the sequences) are depicted as mean values for the control and laminitis groups. T-test showing differential abundance between laminitis and healthy ruminal microbiome (p value < 0.05 or 0.01).

Figure 4

Relative abundances of most abundant phylum levels (near 1% or more in all the sequences) are depicted as mean values for the control and laminitis groups. T-test showing differential abundance between laminitis and healthy ruminal microbiome (p value < 0.05 or 0.01).

Figure 5

Differences between healthy and laminitis ruminal microbiota in alpha diversity (A-E). A: observed species, B: Chao 1, C: ace, D: Shannon, E: Simpson. F: Non-metric multidimensional scaling (NMDS) plot of pair wise Bray-Curtis dissimilarities between all samples processed using the two pipelines. Alpha diversity analysis showing no differential abundance between laminitis and healthy rumen microbiome (p value > 0.05).

Figure 6

The concentration of LPS and lactic acid in the laminitis group obviously increase, while that of histamine in serum remained similar. The values presented are the mean $\pm$ SEM. *P < 0.05 and **P < 0.01 are significantly different from laminitis group.

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