

# Characterization of Bacterial Community of Rumen in Dairy Cows With Laminitis

Jian Gao

Jilin University

Bo Liu

Inner Mongolia Agriculture University: Inner Mongolia Agricultural University

Shuang Li

Jilin University

Ruiying Mu

Linqu Animal Husbandry Bureau

Naisheng Zhang

Jilin University

Xiaoyu Hu

Jilin University

Yunhe Fu (✉ [fuyunhesky@163.com](mailto:fuyunhesky@163.com))

Jilin University <https://orcid.org/0000-0002-6193-2763>

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## Research Article

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## Abstract

**Background:** Laminitis- an inflammation of lamella, could cause great economic loss to dairy industry, which has attracted wide attention around the world. In recent years, microbiota is considered as one of the vital parts that played significant role in various diseases processes. However, current studies are far from sufficient. Aim of this study is to explore the characteristics of ruminal microbiota in laminitis cows.

**Methods:** The serum of bovines with or without laminitis was collected to detect concentrations of lipopolysaccharide (LPS), lactic acid, and histamine, and the ruminal fluid was collected for 16S rRNA sequence analysis.

**Results:** The results showed that a significant increase in LPS and lactic acid levels in laminitis group comparing to control group cows. In addition, the higher abundance of bacteria that -riches acid-enhancing metabolites, namely, *Candidatus Saccharimonas*, *Saccharofermentans*, *Erysipelotrichaceae UCG-009*, *Erysipelotrichaceae UCG-008*, *Clostridium papyrosolvens* and *Ruminococcaceae bacterium AE2021* were detected in the rumen fluid from laminitis bovines.

**Conclusions:** This article confirmed that difference of rumen microbiota were occurred in rumen between health and laminitis bovines. The elevated abundance of bacteria that riches acid- enhancing metabolites, as well as increased the concentration of lactic acid and LPS could be harmful factors to bovines and increase risks of laminitis.

## Background

In the dairy industry, metabolic diseases are main health problems, such as foot disease [1]. Laminitis, an aseptic inflammation of the sensitive lamina of the hoof [2], is one of the key causes to major economic losses[3] and 62% of which contributed by lameness cases [4]. In bovines, it can be categorized as acute, subacute, chronic and subclinical laminitis according to the severity and duration of the incident [5]. In the stage of acute and subacute laminitis, the cows are crippled in all limbs [3]. However, there are only changes in claws in chronic phase without any obvious systemic symptoms. The hoof is elongated into a flattened and broadened shape, named “slipper foot” [6–8] Changes in postures and locomotion are insignificant and also occurs after two to three months of sole ulcers and white-line disease [9, 10]. Although laminitis has been attracting enough attention, its physiopathology remains to be unelucidated[9]. Currently, it is commonly considered as the result of the interaction of various factors, including breeding conditions and metabolic insults, which increases accumulation of toxic substances, such as histamine, lactic acid and LPS [12] leading to vascular lesions and degradation of the suspensory apparatus of the third phalanx within the digit [4, 10, 11].

Host-rumen-microbe interactions play a critical role in the maintenance of physiological activity so as to be a contributing factor to affect the health of dairy cows [12]. Now, it is well established that the disturbance of ruminal microbiota can induce bovine laminitis through indigestion caused by ruminal acidosis. Overuse of the concentrates aiming at increasing the milk yield contributed to the imbalance of

ruminal flora. Increasing harmful bacteria could produce more endotoxins and trigger release of histamine and lactic acid[13–16]. Inflammation reactions are main pathological changes in incipient stage of laminitis and the effects of LPS on it have been examined[17]. In addition, previous research showed that intradermal injection of LPS into bovine induced laminitis [18]. In ruminal acidosis, the release of vasoactive substances (LPS and histamine) leads to vasoconstriction and dilation resulting in the destruction of microvasculature of the corium [22, 23]. The corium- rich in vascular and neurons-nourishes the dermal lamellae between the lamina and the distal phalanx[19, 20]. Although existing researches showed a link between laminitis and lactic acidosis due to carbohydrate overload, we failed to clarify the correlation ship between ruminal microbiota and laminitis. The aim of this paper is to detect the characteristics of rumen flora in cows with laminitis, so as to provide a reference for the development of microecological preparations for treating cow laminitis.

## Materials And 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. At the beginning, animals were selected by lameness examination and divided into health and lameness group. lameness bovines were then further examined by shoeing to identify laminitis [21]. blood samples and rumen fluid were collected respectively with vacutainer tubes with EDTA and with sterile 50 mL centrifuge tubes by rumen puncture [22]. Then samples were kept at -80°C for microbiota analysis. 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.

### Lipopolysaccharide Concentration Detection

Blood was centrifuged at 4000 rpm 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 4000 rpm 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 amply 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 differences between 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.

# Results

## LPS, Lactic Acid, and Histamine in Serum

As shown in Fig. 1A-C, the concentration of LPS and lactic acid in laminitis group significantly increased, while there was no difference in histamine in serum.

## The Composition of Ruminal Bacterial Community

PCR was used to test the V4 region of 16S ribosomal RNA (rRNA) gene of ruminal bacteria and identify characteristics of ruminal bacterial community from sixteen ruminal fluid samples (8 from control and 8 from laminitis). Rarefaction curves displayed that the majority of bacterial diversity had sufficient sequences proved by the sampling depth (see supplementary Fig. S1). Comparing control with laminitis group, there was no significant difference in community richness and diversity observed by species, Chao 1, and ace and Shannon index and Simpson index. Interestingly, the nonmetric multidimensional scaling (NMDS) ordination showed that there was a separation of bacterial community between control and laminitis group using the Bray-Curtis dissimilarity (the value of stress was 0.104) (Fig. 2).

## Changes of Ruminal Bacterial Community at the Phylum Level

At the phylum 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* (34.85% vs 43.64%) were the most abundant phyla in ruminal bacterial community. In addition, the relative abundance of *Proteobacteria* (8.03% vs 2.66%), *Spirochaetes* (1.08% vs 0.48%), *Tenericutes* (1.29% vs 3.65%), *Euryarchaeota* (0.86% vs 1.66%), and

*Cyanobacteria* 0.97% vs 0.95%) was about 1% and more in bovine ruminal microbiota, respectively (As shown in Fig. 3A). The results of T-test showed that the relative abundance of *Tenericutes*, *Saccharibacteria*, and *SR1 (Absconditabacteria)* significantly increased in bovine with laminitis than control group (As shown in Fig. 3B).

### Changes 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 preliminary analysis of the dominant genera are displayed in Fig. 4A and supplementary table 2, *Prevotella 1* (control vs laminitis, 20.44% vs 13.46%), *Succinilasticum* (7.77% vs 7.06%), *Succinivibrionaceae UCG-002* (4.34% vs 0.79%), *Christensenellaceae R-7-group* (4.63% vs 7.94%), *Succinivibrionaceae UCG-001* (2.20% vs 0.30%), *Ruminococcaceae NK4A214-group* (3.52% vs 5.11%), *Ruminococcaceae UCG-014* (2.12% vs 3.29%) and *Rikenellaceae mRC9 gut group* (3.85% vs 4.24%). T-test showed that the relative abundance of *Candidatus Saccharimonas*, *Saccharofermentans*, and *Erysipelotrichaceae UCG-009* significantly increased in laminitis group. Meanwhile, none of differences of the other genera was statistically significant (Fig. 4B).

At the species level, the T-test analysis presented that species of *Clostridium papyrosolvens* and *Ruminococcaceae* bacterium AE2021 significantly increased while the relative abundance of *Prevotella ruminicola*, *Prevotella sp. CA17* and *Treponema saccharophilum* significantly decreased in ruminal microbiota from laminitis samples (Fig. 5A-E). Furthermore, the core genera shared by healthy and laminitis bovine were used to evaluate the link between unique bacterial microbiota and laminitis. The Venn diagram illustrated that the proportion of main genera of healthy group was close to 90.66% and that of laminitis group was responsible for 92.74% (Fig. 5F). These results may explain the relatively positive 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 microbiota community of rumen to prove that theory. At the genus levels, the obvious biomarkers were *Ruminococcaceae UCG 014*, *Candidatus Saccharimonas*, *Saccharofermentans* and *Succinvibrionaceae UCG 002*. While that of species levels was no significant biomarkers (Fig. 6A-B).

## Discussion

Bovine laminitis, one of the most cost of lameness conditions, is an economic drain on producers. It is generally accepted that micro-circulation of blood disorder within the corium induced by microbiota metabolites, such as LPS, lactate, and histamine, is the main pathogenesis of laminitis. Thus, in the present study, we detected the characteristics of rumen microbiota and the concentration of LPS, lactic acid, and histamine in the serum from cows between control and laminitis. The results showed that the concentration of LPS and lactate in serum were increased in the laminitis cows compared to the control cows. In addition, the elevated abundance of bacteria, including *Candidatus Saccharimonas*, *Saccharofermentans*, *Erysipelotrichaceae UCG-009*, *Erysipelotrichaceae UCG-008*, *Clostridium*

*papyrosolvens*, and *Ruminococcaceae bacterium AE2021*, that riches acid-enhancing metabolites could lower the pH of rumen fluid, which leads to the death of gram-negative bacteria and release of endotoxins in rumen. However, the small samples size limits to some extent the generalization of the findings made in this study.

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 ruminal acidosis occurs, plenty of gram-negative bacteria are dead and LPS release. In the early stage of laminitis, inflammation is the main manifestation. LPS is absorbed into blood circulation through the ruminal wall, and then reaches the micro-circulation of the claw. The local LPS has inflammatory effects, such as the activation of cytokines and acute-phase protein release, thrombocytopenia, leukopenia followed by leukocytosis [23]. Besides, recent evidence suggested that pathological changes in inflammation could be made by injection of LPS [18]. In this study, the concentration of LPS significantly increased in the laminitis group compared with the healthy group.

Lactic acid, as vasoactive substances as LPS, are associated with laminitis [24]. Both of them can present a risk to claw deterioration and the laminitis process, including vasoconstriction, dilation, edema and thrombosis, and to interfere with the migration and function of defense cells such as neutrophils[24]. Previous studies reported the increase of histamine and lactic acid in bovine serum during laminitis [4, 5, 24]. Our experiments confirmed previous results and lactic acid in serum of bovine with laminitis went up.

There is an evidence that ruminal bacterial community play a crucial role in pathologic regulation of organism in developing disease [25]. The role of microbial populations received widespread attention across several disciplines in recent years. Therefore, a number of cross-sectional studies suggested an association among microbiota, its metabolites 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 [26]. However, our results showed that there are no significantly difference rumen bacterial phyla between laminitis and control bovines, this may be due to the different diets of different experimental animals. Our study was to collect rumen fluid samples from healthy and laminitis cows with the same diet, whereas other studies have been conducted in animal models of laminitis induced by high carbohydrate feeding.

In the present study, we observed the significant differences in bacterial community between the two groups using NMDS. In brief, compared to healthy group, the genera *Candidatus Saccharimonas*, *Saccharofermentans*, *Erysipelotrichaceae UCG-009*, and *Erysipelotrichaceae UCG-008* increased, most of which were associated with fermentable diets and lower ruminal pH [27–29]. With the reduction of pH, plenty of gram-negative bacteria died and released more LPS. At the species level, *Clostridium papyrosolvens* and *Ruminococcaceae bacterium AE2021* significantly increased. As Boonsaen P argued, *Ruminococcaceae* (e.g. *Ruminococcus flavefaciens* and *Ruminococcus albus*) could produce lactate [30].

These results suggested alteration of ruminal bacterial community may be a factor inducing laminitis by influencing rumen metabolisms, such as the increase of LPS and lactate.

## Conclusions

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

## Abbreviations

LPS: lipopolysaccharide; NMDS: nonmetric multidimensional scaling; LDA: linear discriminant analysis; LEfSe: LDA effect size.

## Declarations

### Ethics approval and consent to participate

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

### Consent for publication

No applicable.

### Availability of supporting data

The data are available upon request.

### Competing interests

The authors declare that they have no competing interests.

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### Author's contributions

J G and B L performed the rumen samples collection and the data analysis. S L performed the LPS, lactic acid, and histamine detection. Tuniyazi. Maimaiti revised the manuscript. X H wrote the manuscript. N Z and Y F designed the study. All authors read, revised and approved the final manuscript.

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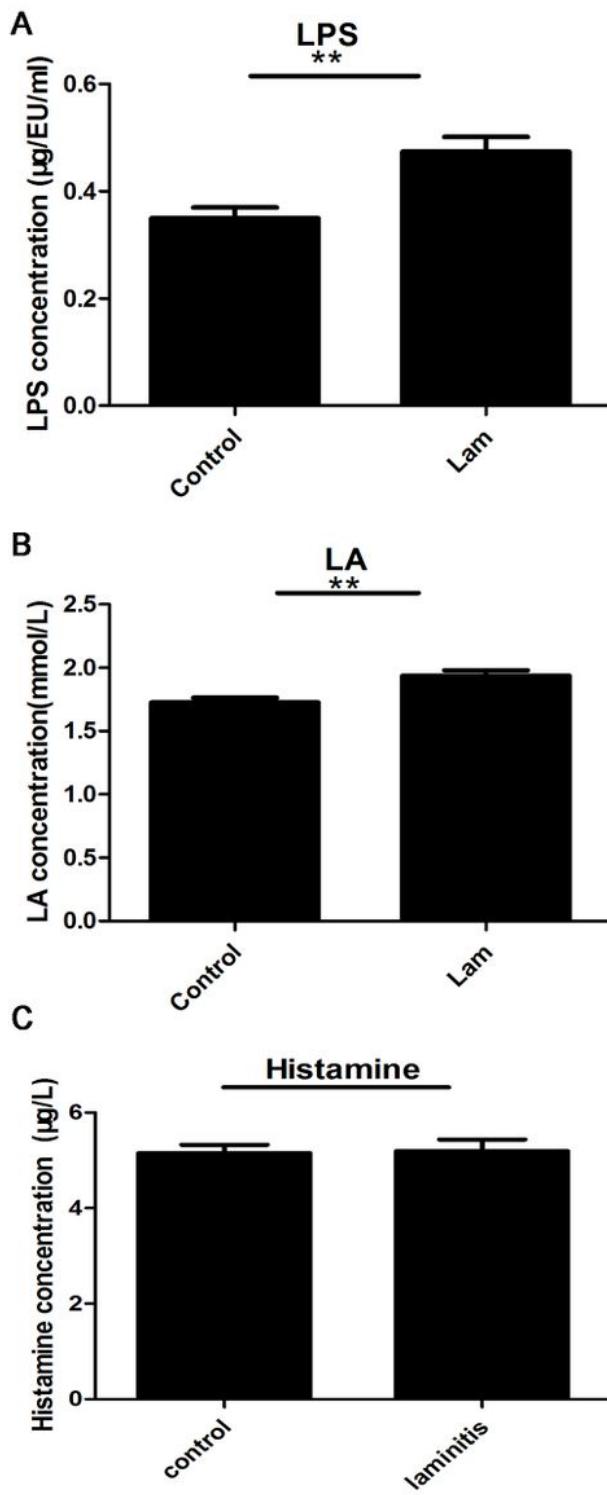
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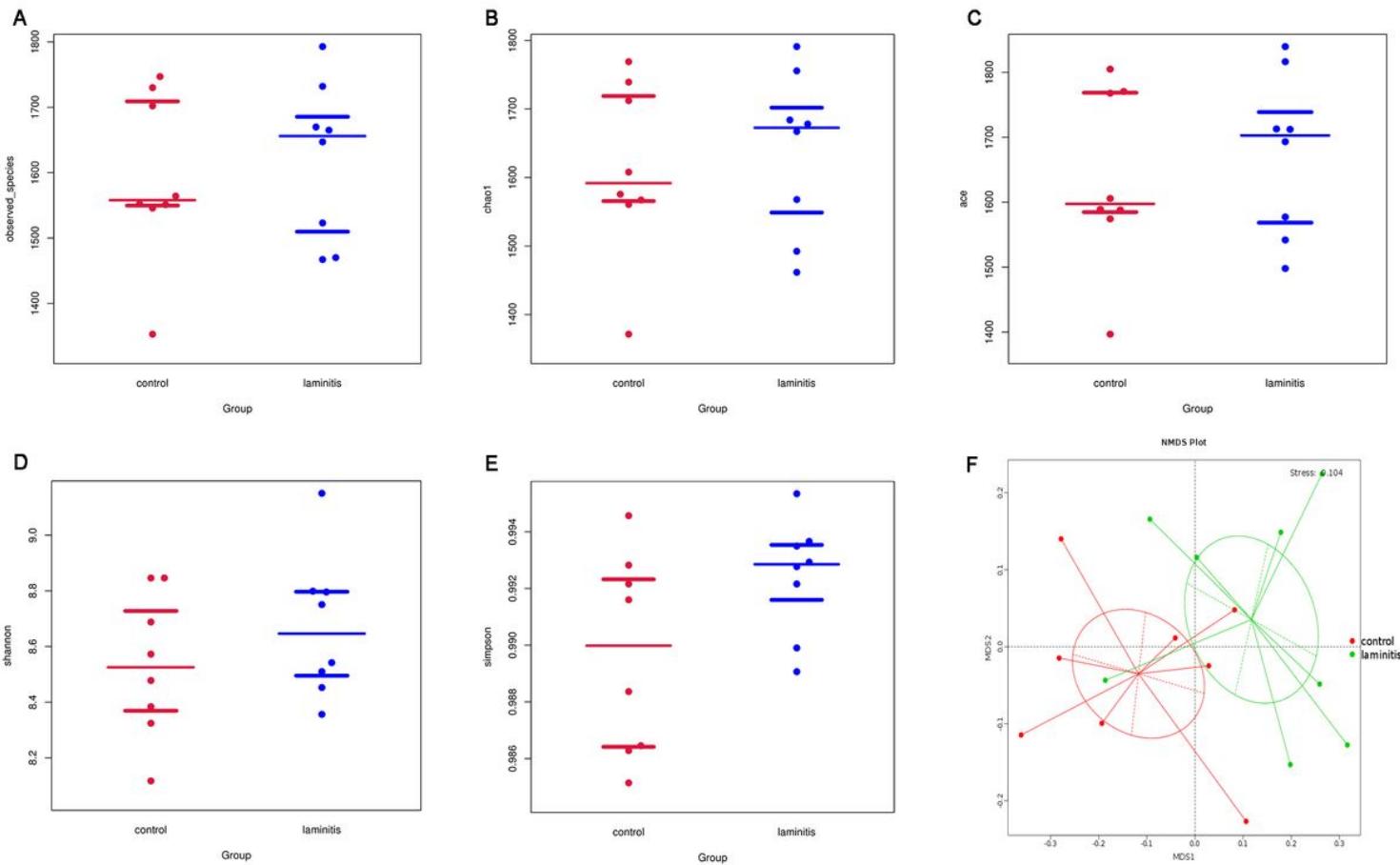
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## Figures



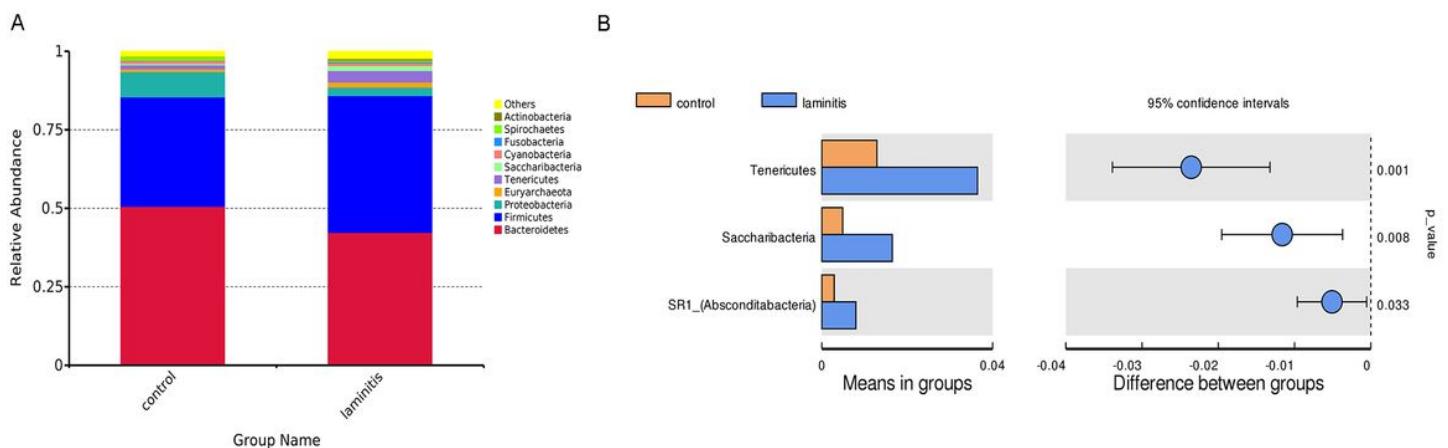
**Figure 1**

LPS, Lactic Acid, and Histamine in Serum. 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.



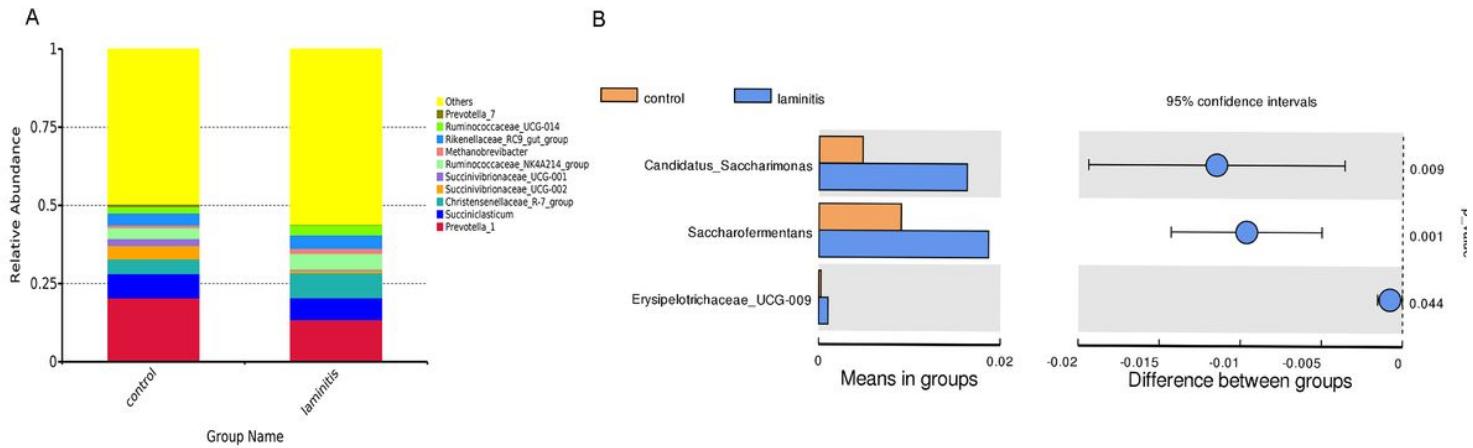
**Figure 2**

The Composition of Ruminal Bacterial Community. 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  $> 0.05$ ).



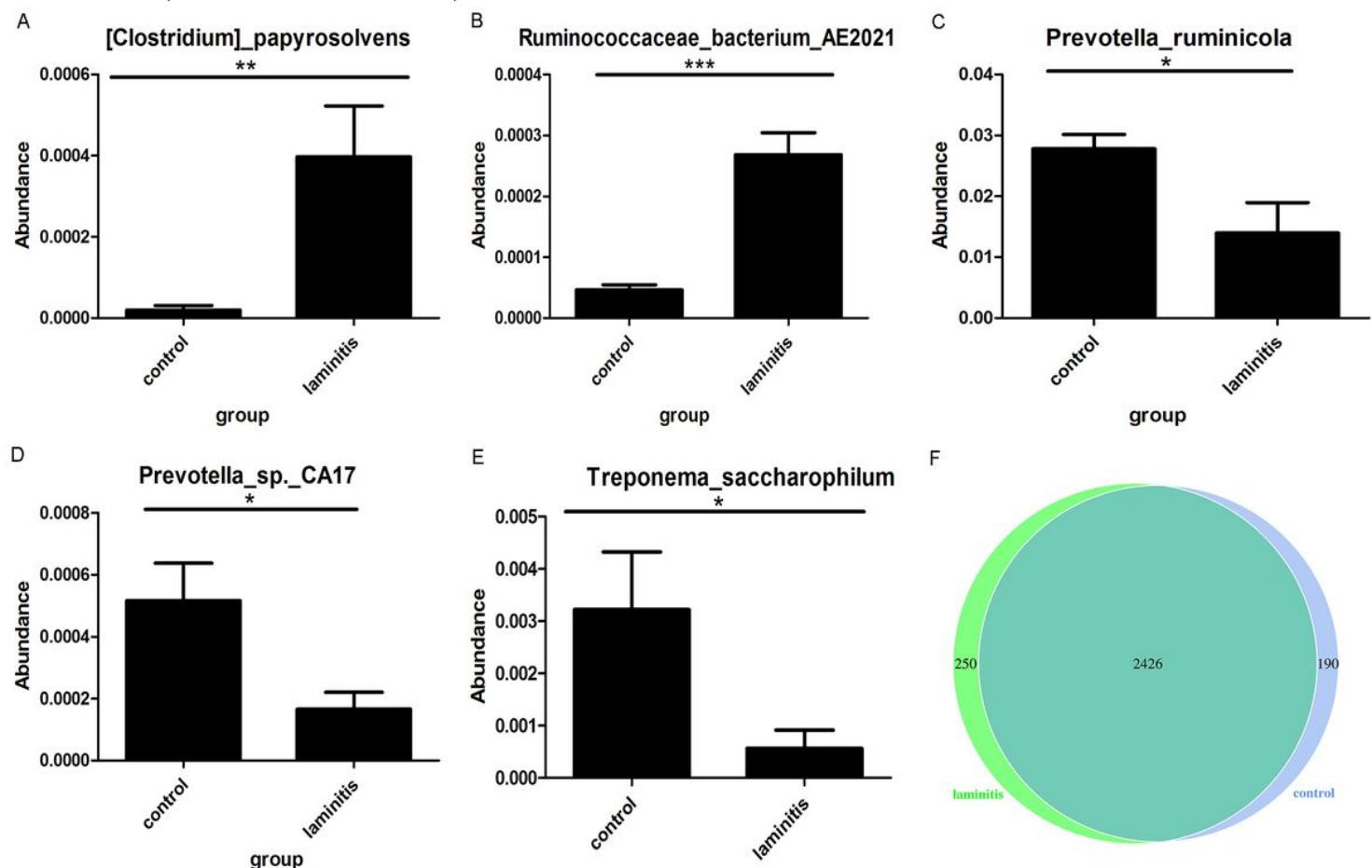
**Figure 3**

Changes of Ruminal Bacterial Community at the Phylum Level. 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  $0.01$ ).



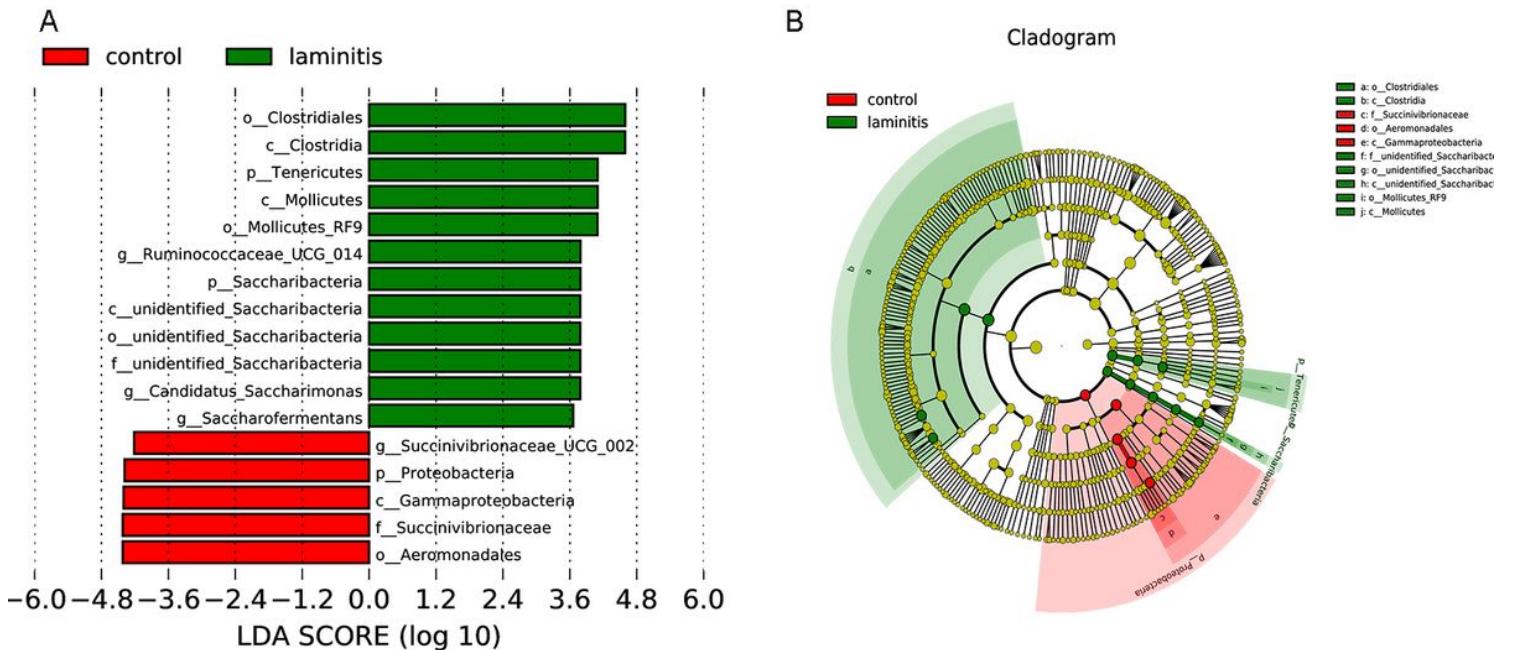
**Figure 4**

Changes of Ruminal Bacterial Community at the Genus Levels. 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 5

Changes of Ruminal Bacterial Community at the Species Levels. 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  $\pm$  SEM. \* $P < 0.05$  and \*\* $P < 0.01$  are significantly different from laminitis group. F: Venn diagram showing the number of core genera in rumen microbiota in laminitis and healthy samples.



## Figure 6

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

## Supplementary Files

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

- Fig.S1.png
- supplementarytable1.xls
- supplementarytable2.xls