Intraruminal infusion of oligofructose alters ruminal microbiota and induces acute laminitis in sheep^{1,2}

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ABSTRACT: The objectives of this study were to examine the clinical response, changes in ruminal bacterial microbiota, and inflammatory response in lamellar tissues during oligofructose-induced laminitis. Ten fistulated sheep were randomly assigned into a control group ($n = 5$) and a treatment group ($n = 5$). The treatment group was infused with oligofructose (21 g/kg BW) by rumen cannula, and the control group was sham-treated with saline. Results showed that all 5 sheep treated with oligofructose developed anorexia and diarrhea 8 to 12 h after the administration of oligofructose. By 12 to 24 h after treatment, the treatment group developed lameness and roach back. Compared with the control group, oligofructose administration decreased ($P < 0.001$) the rumen pH and concentrations of total VFA and increased ($P < 0.001$) the level of lactic acid in the rumen. Microbial data analysis revealed that oligofructose infusion increased the abundance of *Streptococcus* ($P = 0.009$) and *Lactobacillus* ($P = 0.008$) and decreased the percentage of unclassified Christensenellaceae ($P = 0.028$), unclassified Ruminococcaceae ($P = 0.009$), *Butyrivibrio* ($P = 0.016$), unclassified Lachnospiraceae ($P = 0.009$), and

Ruminococcus ($P = 0.009$) compared with the control group. Oligofructose infusion decreased the ACE ($P = 0.047$) and Shannon ($P = 0.009$) indices compared with the control group. The histomorphology analysis revealed that oligofructose overload resulted in damage to the dermoepidermal junction in the lamellar tissue of sheep. Quantitative real-time PCR results showed that compared with the control group, the mRNA expression of membrane-type metalloproteinase-1 ($P = 0.049$) was downregulated whereas the expression of proinflammatory IL-6 ($P = 0.004$) and matrix metalloprotease-9 ($P = 0.037$) was upregulated in the lamellar tissues of the oligofructose treatment group. In general, the present study provides the foundation for a sheep model of oligofructose-overload-induced acute laminitis that could be used in later experiments. Our findings suggest that intraruminal infusion of oligofructose altered ruminal microbiota and resulted in acute laminitis and that the inflammatory damage to the lamellae tissue may be related to the upregulation of matrix metalloprotease-9. The information generated will provide more insight into the systemic effects of lameness caused by oligofructose overload in sheep.

Key words: laminitis, oligofructose, ruminal microbiota, sheep

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INTRODUCTION

Laminitis is defined as a loss of attachment between the inner hoof wall and the distal phalanx, which

causes the bone to be driven downward into the hoof, damaging surrounding arteries and veins and, finally, crushing the corium of the sole and the coronet (Pollitt, 1999). Despite intensive research into laminitis, the etiology, pathogenesis, and triggering factors leading to the disease in ruminants still remain largely unclear (Bailey, 2004; Greenough et al., 2007). In recent decades, 1 model mimicking acute laminitis was successfully tested using an oral dose of oligofructose in cattle (Thoenes et al., 2004). However, the experimental models for cattle lameness were relatively expensive. Sheep are often used as a model for research due to the

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smaller costs associated with the acquisition and slaughter of the animals. Additionally, laminitis decreased welfare and increased economic loss in the sheep industry (Eze, 2002). Therefore, it is necessary to evaluate whether the laminitis model successfully induced by oligofructose overload in cattle can be reproduced in sheep.

Rumen microbial changes in ruminants associated with carbohydrate overload were well documented, such as the proliferation of *Streptococcus bovis* and *Lactobacillus* spp. (Owens et al., 1998; Nagaraja and Titgemeyer, 2007). Even though a model of laminitis induced by oligofructose challenge has been developed in cows (Thoenfer et al., 2004), there is little information about the microbial factors contributing to the development of laminitis induced by oligofructose challenge in ruminants. Previous studies showed that streptococcal species in the hindgut played an integral role in equine laminitis (Bailey, 2004; Al Jassim et al., 2005). If the results from equines also apply to ruminants, it would appear that ruminal microbiota plays an important role in ruminant laminitis induced by carbohydrate overload. Here, we hypothesized that oligofructose overload may alter the rumen microbiota, trigger inflammation in lamellar tissues, and lead to laminitis in sheep. Therefore, the aims of this study were to determine whether alimentary overload with oligofructose would cause laminitis in sheep and to evaluate the effects of oligofructose overload on the alteration of ruminal microbiota and the mRNA expression of genes coding for inflammatory cytokines and matrix metalloproteinase enzymes (MMP) in the lamellar tissue of sheep.

MATERIALS AND METHODS

Animals involved in this study were cared for according to the guidelines of the Nanjing Agricultural University Animal Care and Use Committee. The committee reviewed and approved the experiment and all procedures performed in the present study.

Animal and Experimental Design

A group of 10 male Hu sheep (30.55 ± 0.90 kg BW), fitted with permanent ruminal fistulae, were used in this experiment. All sheep were clinically healthy, with normal locomotion and no history of severe systemic disease or serious claw lesions. The 10 animals were purchased from the same feedlot and were the same age and approximate BW. All animals were offered the same diet, according to 4% of their BW, administered ad libitum. The diets were formulated (Table 1) according to the guidelines for nutrient requirements of 25-kg meat sheep published by the NRC (2007). The animals were fed twice a day, at 0800 and 1800 h, and

were kept in individual pens with free access to water. The experimental period was 36 d, with 30 d used for diet acclimatization, the next 3 d used for oligofructose (Raftilose P95; Beneo-Orafti Inc., Tienen, Belgium) adaptation, and the last 3 d used for the formal experiment. In the last 3 d, we began to continuously sample and then all animals were slaughtered. The 10 animals mentioned earlier were randomly divided into a control group ($n = 5$) and a treatment group ($n = 5$). Oligofructose was dissolved into normal saline (80 g/100 mL of water). On the 31st through 33rd days of the experiment, 5% of the main dose was given as a priming dose twice a day before the formal experiment to gradually allow adjustment of the ruminal microbiota to the new source of carbohydrate. On the 34th day (at 0800 h), the control group was infused with bacteria-free saline at 26.25 mL/kg BW, through the ruminal fistula; the treatment group was infused with 21 g of oligofructose per kilogram of BW, through the ruminal fistula (Thoenfer et al., 2004). On the 36th day (at 0800 h), all sheep were slaughtered to collect samples.

In the present study, the lameness, appetite, and fecal consistency were continuously monitored every 2 h after oligofructose infusion by 1 person, and first time when these signs were observed was recorded. When the sheep developed an arched back or the gait was described as one deliberate step at a time, the sheep was defined as lame (Sprecher et al., 1997). The appetite of sheep was monitored according to whether there was eating or ruminating behavior and the amounts of remaining feed (Danscher et al., 2009). Fecal consistency was visually inspected and divided into 4 grades according to the criterion described in the study of Danscher et al. (2009).

Sample Collection

Samples of ruminal contents were collected before (at 0800 h) and 2, 4, 8, 12, 24, and 48 h after administration of oligofructose via ruminal cannula on the 34th day of the experiment. The ruminal contents were immediately filtered through 4 layers of cheesecloth, the pH value was measured using a mobile pH meter (PB-21; Sartorius AG, Göttingen, Germany), and the filter fluid was collected using an Eppendorf tube to determine the content of VFA and lactic acid. Based on the results of ruminal fermentation parameters in the present study, samples of rumen contents collected at the 12-h time point were used for DNA extraction. At 0800 h on the 36th day of the experiment, all sheep that received oligofructose administration developed laminitis, and then all sheep were slaughtered and claw samples were collected. The distal part of each front limb was separated from the cadavers 4 to 8 min after slaughter and was kept on ice. We used a band saw to harvest a

Table 1. Ingredient and nutrient composition of the experimental diets

Item	Diet
Ingredients composition, % DM	
Oaten hay	50.00
Alfalfa hay	20.00
Corn meal	23.00
Soybean meal	4.00
Limestone	0.50
NaCl	0.50
CaHPO ₄	1.00
Mineral and vitamin supplement ¹	1.00
Total	100.00
Nutrient composition	
Metabolic energy, MJ/kg DM	10.42
CP, % DM	12.83
Crude fat, % DM	2.70
Crude fiber, % DM	21.80
NDF, % DM	43.17
ADF, % DM	27.59
Crude ash, % DM	8.30
Starch, % DM	21.15

¹Contained 16% calcium carbonate, 102 g/kg of Zn, 47 g/kg of Mn, 26 g/kg of Cu, 1,140 mg/kg of I, 500 mg/kg of Se, 340 mg/kg of Co, 17,167,380 IU/kg of vitamin A, 858,370 IU/kg of vitamin D, and 23,605 IU/kg of vitamin E.

horizontally oriented tissue slice from the distal aspect of each claw. First, a cut was made through the long axis of the limb, separating the medial claws from the lateral claws. Second, a horizontal cut removed the sole and the most distal 2 mm of the solar corium to expose the distal surface of the third phalanx. A cut was then made 1.5 cm more proximal from and parallel to the second, and a tissue slice of the distal claw with the sole removed was made. In preparation for later sampling of 2 tissue blocks, radial cuts were made into the slice 1 cm apart, just through the claw wall. Tissue blocks consisting of the claw wall and adjacent soft tissue were then dissected from the third phalanx. Tissue blocks were immediately fixed in 4% neutral-buffered paraformaldehyde and stained with hematoxylin and eosin to observe histology and morphology. Meanwhile, the collected lamellar tissue samples were immediately frozen in liquid nitrogen, and these were used for RNA extraction.

Volatile Fatty Acid and Lactate Determination in Ruminal Fluid

The concentrations of VFA were measured using capillary column gas chromatography (GC-14B; Shimadzu, Inc., Kyoto, Japan; film thickness of the capillary column, 30 m by 0.32 mm by 0.25 mm; 130°C column temperature, 180°C injector temperature, and 180°C detector temperature; Qin, 1982). In brief, 10 mL of filtered ruminal fluid was added to 2 mL

of 25% (wt/vol) metaphosphate crotonic acid, supernatant fluid was stored at –20°C for VFA determination, and then VFA in the ruminal fluid were measured using a capillary column gas chromatograph. The concentration of lactic acid was determined according to the method described by Barker and Summerson (1941).

Ruminal Microbial DNA Extraction, PCR Amplification, Illumina MiSeq Sequencing, and Sequencing Data Processing

Rumen contents (0.3 g) collected 12 h after oligofructose infusion were used for DNA extraction. The DNA was extracted by a bead-beating method using a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, the rumen contents were disrupted in an ASL buffer (Qiagen, Hilden, Germany) and homogenized with 100 mg of zirconium beads (0.1 mm) in a Mini-Beadbeater-1 (Biospec Products, Inc., Bartlesville, OK) at a rate of 4,800 rpm/min 4 times for 30 s each time at room temperature. Lysozyme was then added at a final concentration of 20 mg/mL (Sigma-Aldrich Inc., Darmstadt, Germany), and the suspension was incubated at 37°C for 40 min to improve lysis efficiency. Subsequently, the mixture was incubated in a 95°C water bath for 5 min to further increase the amount of total DNA extraction. The DNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Minneapolis, MN). The V3 through V4 region of the bacteria 16S rRNA gene was amplified by PCR (95°C for 2 min followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min) using primers 338F (5'-barcode-ACTCCTRCGGGAGGCAGCAG-3') and 806R (5'-GGACTACCVGGGTATCTAAT-3'; amplicon length approximately 470 bp), where the barcode was a base-8 sequence unique to each sample. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen BioScience, Union City, CA) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega Corp., Madison, WI). Polymerase chain reaction production from each sample was used to construct a sequencing library with an Illumina TruSeq DNA Sample Preparation Kit (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. An Illumina TruSeq PE Cluster Kit and TruSeq SBS Kit (Illumina Inc.) were used to perform cluster generation, template hybridization, isothermal amplification, linearization, blocking, and denaturation and hybridization of the sequencing primers. Paired-end sequencing 2 × 250 bp was performed to sequence all libraries on an Illumina MiSeq platform (Illumina Inc.) according to the standard protocols (Caporaso et al., 2012).

Sequences from the Illumina MiSeq platform were processed using the QIIME (version 1.70) software package (Campbell et al., 2010). Quality filtering was performed with the following criteria: 1) the 250-bp reads were truncated at any site receiving an average quality score < 20 over a 10-bp sliding window, discarding the truncated reads that were shorter than 50 bp; 2) exact barcode matching, 2-nucleotide mismatch in primer matching, and reads containing ambiguous characters were removed; and 3) only sequences that overlapped longer than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded. Operational taxonomic units (OTU) were clustered with a 97% similarity cutoff using UPARSE (version 7.1; <http://drive5.com/uparse/>; accessed August 10, 2016), and chimeric sequences were identified and removed using UCHIME (Edgar, 2010). The most abundant sequences within each OTU were designated “representative sequences,” and 1,911 representative sequences were then aligned against the SILVA database (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010) with the default parameters set by QIIME. A PH Lane mask supplied by QIIME was used to remove the hypervariable regions from the aligned sequences. Operational taxonomic units were identified using 80, 95, or 97% similarity as criteria for assigning sequences to the same phyla, genera, and species, respectively. FastTree (Price et al., 2009) was used to create a phylogenetic tree of the representative sequences. Sequences were classified using the Ribosomal Database Project classifier with a standard minimum support threshold of 80% (Wang et al., 2007). Community diversity was estimated using the abundance-based coverage estimator (ACE), Chao1, and Shannon indices. The unweighted UniFrac distance method was used to perform a principal coordinate analysis (Lozupone and Knight, 2005), and an unweighted distance-based analysis of molecular variance (AMOVA) was conducted to assess the significant differences among the samples using the mothur program (version 1.29; Schloss et al., 2009). The raw sequence files are available from the Sequence Read Archive of the National Center for Biotechnology Information under the accession number SRP106853.

Ribonucleic Acid Isolation, Primer Design, and Real-Time PCR

Total RNA was extracted from the lamellar tissue samples using TRIzol (Takara Bio Inc., Otsu, Japan) as described by Chomczynski and Sacchi (1987). The RNA concentration was then quantified using a NanoDrop spectrophotometer (ND-1000UV-Vis; Thermo Fisher Scientific, Madison, WI). The absorption ratio (260:280 nm) of all samples was between 1.8

and 2.0, indicating high RNA purity. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose–formaldehyde gel to verify integrity. The concentration of RNA was adjusted to 1 µg/µL based on optical density and stored at –80°C. The total RNA (1 µg) was reverse-transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio Inc.), carefully following the manufacturer’s instructions.

The primers for IL-1β, IL-10, TNF-α, IFN-γ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used were described by Liu et al. (2013; Table 2). The primer sets were designed to recognize and amplify the conserved nucleotide sequences that encode sheep MMP-2, MMP-9, membrane type-1 MMP (MT1-MMP), and tissue inhibitors of metalloproteinases-1 (TIMP-1; Pierson, 2007). The primers used in the present study are shown in Table 2. All of the primers were synthesized by Invitrogen Life Technologies (Shanghai, P.R. China). Real-time quantitative PCR of the target genes and GAPDH were performed using the ABI 7300 real-time PCR system (Applied Biosystems Inc., Foster City, CA) with fluorescence detection of Synergy Brands Green dye (Takara Bio Inc., Otsu, Japan). Amplification conditions were as follows: 95°C for 30 s followed by 40 cycles of 5 s at 95°C and 34 s at 64°C. Each sample contained 1 to 10 ng of cDNA in 2x SYBR Green PCR Master Mix (Takara Bio Inc.) and 200 nmol/L of each primer in a final volume of 20 µL. All measurements were performed in triplicate. A reverse-transcription-negative blank of each sample and a no-template blank served as negative controls. The relative amount of each studied mRNA was normalized to *GAPDH* mRNA levels as a housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

The dynamic data on ruminal pH, VFA, and lactic acid were analyzed using the GLM procedure of SPSS (SPSS version 20; SPSS Inc., Chicago, IL). The independent sample *t*-test procedure (SPSS version 20) was used to analyze the real-time PCR data. The microbial data were analyzed using the Kruskal–Wallis test procedure (SPSS version 20). The significance was declared at $P < 0.05$.

RESULTS

Clinical Observations

In the present study, all 5 sheep developed anorexia and diarrhea 8 to 12 h after receiving oligofructose, and the anorexia and diarrhea lasted for at least 28 h after the first observance of those symptoms. These sheep also

Table 2. Primers for sequences and gene identifier (ID) for all gene transcripts analyzed by quantitative real-time PCR

Gene name ¹	Forward and reverse sequences ²	Gene ID	Amplicon size, bp
<i>IL-1β</i>	F: CATGTGTGCTGAAGGCTCTC R: AGTGTCGGCGTATCACCTTT	Liu et al. (2013)	173
<i>IL-6</i>	F: CCAATCTGGGTTCAATCAGG R: ACCCACTCGTTTGAGGACTG	Liu et al. (2013)	241
<i>IL-10</i>	F: TTAAGGGTTACCTGGGTTGC R: CCCTCTCTTGAGCATATTGA	Liu et al. (2013)	239
<i>TNF-α</i>	F: CAAGTAACAAGCCGGTAGCC R: AGATGAGGTAAAGCCCGTCA	Liu et al. (2013)	155
<i>IFN-γ</i>	F: TGATTCAAATTCGGTGGAT R: GCAGGCAGGAGAACCATTAC	Liu et al. (2013)	166
<i>MMP-2</i>	F: GACCAGAGCACCATTGAGACC R: CACGAGCGAAGGCATCATCC	XM_005691985.3	143
<i>MMP-9</i>	F: TGAGGGTAAGGTGCTGCTGTTC R: AAGATGTCGTGCGTGCTAATGG	NM_001314269.1	74
<i>TIMP-1</i>	F: AGGACCAAACTCACCTGGC R: GTGGGTTCTCTGGTGTAGCC	NM_001285765.1	74
<i>MT1-MMP</i>	F: AGCACTGGGTGTTTGAGGAA R: GCATCCAGAAGAGAGCAGCA	NM_001285765.1	111
<i>GADPH</i>	F: GGGTCATCATCTCTGCACCT R: GGTCTATAAGTCCCTCCACGA	Wang et al. (2009)	180

¹*IL-1 β* = interleukin-1 beta; *IL-6* = interleukin-6; *IL-10* = interleukin-10; *TNF- α* = tumor necrosis factor alpha; *IFN- γ* = interferon-gamma; *MMP-2* = matrix metalloproteinase-2; *MMP-9* = matrix metalloproteinase-9; *TIMP-1* = tissue inhibitor of metalloproteinase-1; *MT1-MMP* = membrane type-1 matrix metalloproteinase enzyme.

²F = forward; R = reverse.

developed lameness and roach back due to foot pain by 12 to 24 h after oligofructose administration, whereas the control sheep did not, and the lameness in these sheep did not resolve at any time before slaughter after the onset of the challenge. The clinical signs observed in sheep given oligofructose indicate that all animals receiving oligofructose developed laminitis.

Ruminal Fermentation Parameters

In the current study, diurnal data showed that oligofructose-treated sheep had a lower ruminal pH compared with the control group ($P < 0.001$; Fig. 1A). The ruminal pH was also affected by the sampling hour ($P < 0.001$). Starting almost 4 h after oligofructose administration and continuing until the sampling at 24 h, the average ruminal pH was below 5.0 in the oligofructose treatment group, which was lower than that of the control group. The diurnal data showed that sheep that received oligofructose had a higher level of lactate compared with the control group ($P < 0.001$; Fig. 1B). Ruminal lactic acid concentration was also affected by the sampling time ($P < 0.001$), which was increased during 0 to 12 h and reached the maximum at 12 h after oligofructose infusion and then continually decreased.

Diurnal variations of ruminal acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, total VFA, and acetate-to-propionate ratio are displayed in Fig. 2.

Starting almost 8 h after oligofructose administration and continuing until the sampling at 24 h, the concentrations of acetate ($P < 0.001$), propionate ($P < 0.001$), butyrate ($P < 0.001$), isobutyrate ($P < 0.001$), and isovalerate ($P = 0.002$) as well as the total VFA ($P < 0.001$) were lower than in the control group, whereas the level of valerate ($P = 0.004$) and the ratio of acetate to propionate ($P < 0.001$) increased. A significant interaction between group and time was observed in most cases, with the exception of isobutyrate.

Changes in the Composition of Ruminal Microbiota

In total, 301,112 high-quality reads, accounting for 0.97 of their raw reads, were available for analysis. In the current study, a total of 18 phyla were detected (Table 3). At the genus level, these sequences were assigned to 150 different genera. The rarefaction curves of ruminal bacterial communities are shown in Fig. 3A. At the OTU level, the rarefaction analysis revealed that the sequencing depths of the samples in the present study could meet the analysis requirements. Compared with the control group, the ACE index (Fig. 4A), Shannon index (Fig. 4B), and OTU number (Fig. 4D) were lower ($P < 0.05$) in treatment group. No significant difference was observed in the Chao1 index ($P = 0.117$) between the 2 groups (Fig. 4C). To compare the structure of the bacterial community across all samples, the unweighted

Figure 1. Diurnal variation of ruminal pH and the concentration of lactic acid in the rumen fluid of the control (○; $n = 5$) and the oligofructose treatment groups (■; $n = 5$). The value is depicted as means $\pm$ SEM ($n = 5$). A) The dynamic change of pH value over 48 h after administration oligofructose. B) The ruminal lactic acid concentration change over 48 h after oligofructose administration.

Figure 2. Diurnal variation of the concentration of acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, and total VFA as well as acetate-to-propionate ratio in the rumen fluid of the control (○; $n = 5$) and the oligofructose treatment groups (■; $n = 5$). The value is depicted as means $\pm$ SEM ($n = 5$).

Table 3. Effects of oligofructose (Oligo) overload on average relative abundance¹ of phylum level (% of total sequences) in rumen content

Phylum	Abundance, %		SEM	P-value
	Control	Oligo		
Firmicutes	50.57	80.43	0.064	0.028
Bacteroidetes	43.57	14.41	0.061	0.016
Actinobacteria	1.84	1.60	0.005	0.754
Spirochaetes	1.02	1.18	0.003	0.347
Proteobacteria	0.82	1.32	0.002	0.251
Tenericutes	0.50	0.22	0.001	0.076
Candidate division TM7	0.45	0.12	0.001	0.175
Fibrobacteres	0.45	0.15	0.001	0.028
SHA-109	0.34	0.10	0.001	0.753
Cyanobacteria	0.22	0.13	<0.001	0.402
Elusimicrobia	0.05	0.14	<0.001	0.076
Synergistetes	0.04	0.05	<0.001	0.046
Verrucomicrobia	0.04	<0.01	<0.001	0.528
Candidate division SR1	0.03	<0.01	<0.001	0.916
Lentisphaerae	0.03	0.10	<0.001	0.637
Unclassified bacteria	0.03	0.03	<0.001	0.005
Chloroflexi	0.01	0.01	<0.001	0.396
Fusobacteria	<0.01	0.02	<0.001	0.013

¹Values are represented by means; $n = 5$.

UniFrac metric was used to assess β -diversity. The principle coordinates analysis (PCoA) profile showed that the samples from the control group were distinctly separate from those from the treatment group (Fig. 3B). Principle coordinates analysis also revealed that PCoA axis 1 accounted for 49.29% of the variation, whereas PCoA axis 2 accounted for 10.26%. A significant difference was found in the microbial communities between the 2 groups (AMOVA; $P < 0.05$).

At the phylum level, the phyla Firmicutes and Bacteroidetes together represented more than 90% of the ruminal microbial community in the 2 groups (Table 2). Among the 18 phyla detected in the present study, the relative abundance of Firmicutes, Bacteroidetes, Fibrobacteres, Synergistetes, Fusobacteria, and unclassified bacteria was affected by treatment. Among them, oligofructose overload decreased the abundance of the phyla Bacteroidetes ($P = 0.016$) and Fibrobacteres ($P = 0.028$) and the unclassified bacteria ($P = 0.005$) and increased the percentage of phyla Firmicutes ($P = 0.028$), Synergistetes ($P = 0.046$), and Fusobacteria ($P = 0.013$) compared with the control group.

At the genus level, 150 genera were detected in the present study. For clarity and visualization purposes, the predominant genera (with an average relative abundance of $>1\%$) are presented in Fig. 5. In comparison with the control group, oligofructose overload increased the proportion of *Streptococcus* ($P = 0.009$) and *Lactobacillus* ($P = 0.008$) while decreasing the

Figure 3. Sample-based rarefaction analysis curves and principal coordinate analysis of microbial diversity across all samples using an unweighted UniFrac matrix. A) The rarefaction curves across all samples. Each curve represents 1 individual sample. B) Principal coordinate analysis of microbial diversity across all samples. The percentage of variation explained by principal components 1 (PC1) and principal components 2 (PC2) are indicated in the axis. C = the control samples ($n = 5$); T = the treatment samples ($n = 5$). The control group and the treatment group were clustered together on the PC1 axis.

abundance of *Butyrivibrio* ($P = 0.016$), *Ruminococcus* ($P = 0.009$), and some unclassified groups, including unclassified Bacteroidales ($P = 0.009$), unclassified Prevotellaceae ($P = 0.009$), unclassified Rikenellaceae ($P = 0.028$), unclassified Christensenellaceae ($P = 0.009$), unclassified Ruminococcaceae ($P = 0.009$), and unclassified Lachnospiraceae ($P = 0.028$). There was no significant difference ($P > 0.05$) in the percentage of *Quinella*, *Acetitomaculum*, or *Succiniclasicum* between the 2 groups.

At the OTU level (Table 4), OTU1650 (belonging to genus *Streptococcus* and phylum Firmicutes) was the most abundant OTU (48.16% of total sequences) in the oligofructose treatment group, whereas OTU394 (*Quinella*) was predominant in the control group. Compared with the control group, oligofructose

Figure 4. Estimated microbial diversity in the control (●; $n = 5$) and oligofructose treatment groups (■; $n = 5$). The observed abundance-based coverage estimator (ACE) index (A), Shannon index (B), or Chao index (C) or operational taxonomic unit (OTU) numbers (D) for the control and the oligofructose treatment group are shown. The data are presented as means $\pm$ SEM and the long line represents the mean value of the group; the two short lines represent error bars. The Kruskal–Wallis H(K) nonparametric test was used to calculate significance between the 2 groups.

overload increased the abundance of OTU1650 (*Streptococcus*; $P = 0.009$), OTU1244 (*Quinella*; $P = 0.015$), OTU624 (*Quinella*; $P = 0.044$), and OTU1916 (*Lactobacillus*; $P = 0.008$) while decreasing the percentage of OTU748 (unclassified Ruminococcaceae; $P = 0.028$), OTU1061 (unclassified Christensenellaceae; $P = 0.016$), OTU1464 (*Butyrivibrio*; $P = 0.028$), OTU1683 (unclassified Rikenellaceae; $P = 0.016$), and OTU1554 (unclassified Prevotellaceae; $P = 0.047$).

Histopathological Examination of Claw

Representative light micrographs of cross sections of the lamellar tissue from the 2 groups are shown in Fig. 6A and 6B. In the control group, the dermal and epidermal lamellae were tightly interlocked. The tips of the epidermal lamellae (arrow) were normally smooth and rounded (Fig. 6A). In sheep infused with oligofructose, the inner part of epidermal lamellae appeared stretched and the epidermal lamellae were thin and irregular (Fig. 6B). The lamellar tissue had been damaged in the oligofructose treatment group compared with the control group.

Gene Expression of Cytokines and Matrix Metalloproteinase

The mRNA expressions of cytokines are shown in Fig. 6C and 6D. No significant difference ($P > 0.05$) was observed in the expression of *IL-1 β* , *IL-10*, *TNF- α* , *IFN- γ* , tissue inhibitors of metalloproteinases (*TIMP*), and *MMP-2* genes in the lamellae tissue between the 2 groups. Compared with the control group, oligofructose administration increased the relative mRNA expression of *IL-6* ($P = 0.004$) and *MMP-9* ($P = 0.037$) and decreased the expression of the *MT1-MMP* gene ($P = 0.049$).

DISCUSSION

In recent years, laminitis has been successfully induced by oligofructose overload in equines (van Eps and Pollitt, 2006, 2009). Thoenes et al. (2004) found that acute bovine laminitis can be induced by alimentary oligofructose overload and that laminitis animals first developed depression, diarrhea, and anorexia and then developed transient fever, severe metabolic acidosis, and moderate dehydration. In line with these reports, the present study showed that a direct association between oligofructose infusion and clinical signs of acute laminitis was demonstrated in all 5 animals infused with oligofructose, whereas no animals in the

Figure 5. Relative abundances of most abundant genus levels (>1% all the sequences) are depicted as mean values for the control and oligofructose treatment groups. * $P < 0.05$; ** $P < 0.01$.

control group could be classified as having laminitis. The oligofructose-treated sheep developed diarrhea and stopped eating after 8 h of infusion. This situation lasted for about at least 28 h, when eating was recovered and diarrhea was alleviated. In addition, the oligofructose-treated sheep displayed lameness and the roach back phenomenon; we also found that the lameness was mostly observed in the front hooves of the sheep. Therefore, we concluded that we developed an inexpensive experimental model for the study of acute laminitis in ruminants.

It is well known that laminitis has been strongly associated with rumen acidosis induced by a high content of easily fermented carbohydrates in cattle. Danscher et al. (2009) reported that an oral dose of oligofructose (17 g/kg BW) induced ruminal acidosis in dairy heifers. Thoenes et al. (2004) also found that alimentary oligofructose overload caused acute rumen acidosis in 6 dairy heifers. In this study, the rumen pH was less than 5.0 after 4 h of infusion, suggesting that acute rumen acidosis was induced in oligofructose-treated sheep. In addition, ruminal lactate con-

centrations were immediately increased in sheep after oligofructose infusion, and the increase in lactate concentrations occurred concurrently with a decrease in VFA, indicating that ruminal lactate played a key role in the occurrence of acute ruminal acidosis and that the systemic impact of acute acidosis might contribute to induce the laminitis (Nocek, 1997).

In the present study, our results revealed that the administration of oligofructose strongly altered the composition and structure of the microbiota in the rumen of sheep, as reflected by the clustering of samples using PCoA. In general, oligofructose overload increased the proportion of phylum Firmicutes and genera *Streptococcus* and *Lactobacillus* whereas it decreased the abundance of phyla Bacteroidetes and Fibrobacteres and genera *Butyrivibrio* and *Ruminococcus* compared with the control group. *Streptococcus* and *Lactobacillus* (belonging to the phylum Firmicutes) were the predominant amylolytic, amylodextrin-, and maltose-utilizing bacteria in the rumen of sheep, and these species could rapidly proliferate by utilizing starch or soluble sugars and then produce lactate (Nagaraja and Titgemeyer, 2007).

Table 4. Effects of oligofructose (Oligo) overload on average relative abundance of top 30 operational taxonomic units (OTU; % of total sequences) in rumen content¹

Phylum	Genus	OTU ID ²	Abundance, %		SEM	P-value
			Control	Oligo		
Firmicutes	Unclassified Ruminococcaceae	OTU748	3.45	0.70	0.88	0.028
	Unclassified Christensenellaceae	OTU1061	2.45	0.76	0.34	0.016
		OTU864	1.48	0.67	0.36	0.251
		OTU712	1.67	0.04	0.77	0.280
		OTU123	0.27	1.23	0.51	1.000
	<i>Succiniclasticum</i>	OTU1537	0.77	0.18	0.27	0.249
	<i>Streptococcus</i>	OTU1650	0.03	48.16	9.24	0.009
	<i>Quinella</i>	OTU394	6.74	0.18	3.13	0.465
		OTU1244	0.02	5.57	2.72	0.015
		OTU624	<0.01	1.22	0.60	0.044
	<i>Lactobacillus</i>	OTU1916	<0.01	3.09	3.90	0.008
	<i>Butyrivibrio</i>	OTU1464	5.66	0.41	1.76	0.028
		OTU1342	1.23	0.44	0.24	0.117
		OTU310	1.15	0.24	0.32	0.076
	<i>Acetitomaculum</i>	OTU379	2.06	0.55	0.74	0.834
		OTU1159	0.34	1.39	0.51	0.602
Bacteroidetes	Unclassified Rikenellaceae	OTU1683	1.18	0.20	0.24	0.016
	Unclassified Prevotellaceae	OTU753	1.94	0.05	0.95	0.638
		OTU1554	1.00	0.05	0.39	0.047
	Unclassified Bacteroidales	OTU378	1.09	0.07	0.41	0.911
	<i>Prevotella</i>	OTU1423	5.71	<0.01	2.85	0.057
		OTU915	0.90	1.98	0.89	0.525
		OTU1237	2.04	0.08	0.93	0.347
		OTU1886	0.38	1.04	0.45	0.525
		OTU1196	1.27	0.10	0.61	0.281
		OTU532	1.19	0.08	0.49	0.916
		OTU996	0.44	0.63	0.20	0.675
		OTU517	1.05	0.01	0.49	0.293
		OTU659	0.77	0.22	0.33	0.746
	Unclassified Coriobacteriaceae	OTU1178	0.14	0.95	0.32	0.916

¹Values are represented by means; $n = 5$.²ID = Identifier.

Therefore, in the present study, the accumulation of ruminal lactate in sheep that received oligofructose administration may be the result of an increase in the proportion of genera *Streptococcus* and *Lactobacillus*. In addition, the increase in ruminal *Streptococcus* and *Lactobacillus* were also considered to be a typical feature of acute ruminal acidosis in ruminants (Schwartzkopf-Genswein et al., 2003; Nagaraja and Titgemeyer, 2007). Some species in genera *Butyrivibrio* and *Ruminococcus* (belonging to the phylum Bacteroidetes) and Fibrobacteres are known to be fibrolytic organisms (Fernando et al., 2010; Khafipour et al., 2016), and a decrease in these organisms in the rumen of sheep that received oligofructose administration may be linked with the lower ruminal pH, because it is known that these fiber-degrading bacteria are sensitive to low pH (Russell and Wilson, 1996).

Currently, the widely accepted hypothesis on laminitis occurrence is that laminitis is caused by reduced

blood flow to the lamellae, with the subsequent necrosis resulting in lamellar hypoxia, thereby initiating the development of laminitis (Coffman et al., 1970; Garner et al., 1975; Yelle, 1986; Hinckley, 1996). This hypothesis was confirmed by the findings that vasoactive amines (histamine, tryptamine, and phenylethylamine), produced by the decarboxylation of AA by bacteria genera *Lactobacillus* and *Streptococcus* in the hindgut of equines (Bailey et al., 2002, 2003a,b), caused reduction of digital blood flow and were likely responsible for triggering digital vasoconstriction (Bailey, 2004). Takahashi and Young (1981) also found that the prolonged action of histamine in the hoof caused severe tissue damage, leading to the development of bovine laminitis. As mentioned earlier, the current study showed that the proportion of genera *Streptococcus* and *Lactobacillus* significantly increased in the rumen of oligofructose-treated sheep. In addition, the increase in

Figure 6. Administration of oligofructose effects on the mRNA relative expression of inflammatory factors and matrix metalloproteinases and sheep lamellar layer histology. PDL = primary dermal lamellae; SDL = secondary dermal lamellae; SEL = secondary epidermal lamellae; ns = no significance. Panels C and D represent the effect of oligofructose administration on inflammatory factors and matrix metalloproteinases mRNA relative expression in lamellar tissue using quantitative real-time PCR. In the figure, the values of the control group ($\square$; $n = 5$) and the treatment group ($\blacksquare$; $n = 5$) are represented as mean $\pm$ SEM. (A) Hematoxylin and eosin stain. The control group was normal appearance of the inner part of the lamellae layer (bar = 100 μ m). The tips of the epidermal lamellae (arrow) are normally smooth and round. (B) Hematoxylin and eosin stain. The treatment group was abnormal appearance of the inner part of the lamellae layer. IL-1 β = interleukin-1beta; IL-6 = interleukin-6; IL-10 = interleukin-10; TNF- α = tumor necrosis factor; IFN- γ = interferon-gamma; MMP-2 = matrix metalloproteinase-2; MMP-9 = matrix metalloproteinase-9; TIMP-1 = tissue inhibitor of metalloproteinase-1; MT1-MMP = membrane type-1 matrix metalloproteinase; TIMP = tissue inhibitors of metalloproteinases.

ruminal *Streptococcus* and *Lactobacillus* were also considered to be a typical feature of acute ruminal acidosis induced by a starch challenge in cattle (Schwartzkopf-Genswein et al., 2003; Nagaraja and Titgemeyer, 2007). Therefore, our findings, combined with the reports of equine and bovine laminitis and acute ruminal acidosis in cattle, suggest that *Streptococcus* spp. and *Lactobacillus* spp. may be involved in the series of events that precedes the onset of laminitis in ruminants.

Lameness is believed to be related to the increase in the mRNA expression of a number of proinflammatory molecules such as IL-1 β (Fontaine et al., 2005) and TNF- α in the lamellar tissue of equines (Rodgers et al., 2001). The mechanism underlying the relationship is that proinflammatory cytokines can lead to lamellar organism edema, causing laminitis. In the equine laminitis model induced by black walnut extract or oligofructose, the researchers observed that the mRNA expression of IL-1 β , IL-6, and TNF- α increased in lamellar tissues (Rodgers et al., 2001). The current study showed that alimentary oligofructose overload increased the expressions of IL-6 in the

lamellar tissue of sheep, which is in line with previous studies (Belknap et al., 2007).

Matrix metalloproteinase enzymes, a family of zinc-dependent proteinases, were believed to be involved in the physiological and pathological degradation of the extracellular matrix in lamellar tissues (Kleiner and Stetler-Stevenson, 1999). It was also believed that its activities are controlled by TIMP (Murphy, 2011). The MMP-2 and MMP-9 enzymes are metalloproteases capable of degrading type IV collagen, which is a major component of the hoof's lamellar basement membrane. MMP-2 is secreted as an inactive proenzyme and requires interaction with MT1-MMP for its activation (Seiki, 1999). Previous studies revealed that alimentary carbohydrate (starch or oligofructose) overload models of laminitis in equines have been associated with evidence of increased MMP-9 activity in lamellar tissues (Mungall et al., 2001; Wang et al., 2014). In line with these reports, our data revealed that primary epidermal lamellae became shorter and sharper, the cell matrix was degraded, and the relative expressions of MMP-9 related to the degradation of

the extracellular matrix increased in the oligofructose treatment group. These results imply that the elevated presence of MMP-9 may contribute to pathology during the occurrence of laminitis in sheep. In the current study, the reason the relative expression of MT1-MMP declined in the oligofructose treatment group was not clear and requires further investigation.

Conclusion

In conclusion, the current study showed that oligofructose overload altered ruminal fermentation and microbiota, especially causing an increase in the relative abundance of *Lactobacillus* and *Streptococcus*, and led to lactate accumulation and a lower ruminal pH. Oligofructose overload also activated the inflammation response and the MMP family and increased the expression of the proinflammatory molecules IL-6 and MMP-9. In general, we conclude that oligofructose overload is able to induce signs of acute laminitis in sheep, in which lameness is the vital signal, and that the disturbance of ruminal microbiota played an important role in the onset of laminitis. Therefore, this inexpensive animal model can be used to investigate the pathogenesis and pathophysiology of ruminant laminitis in the future.

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