Comparison of Fecal Microbiota between German Holstein Dairy Cows with and without Left-Sided Displacement of the Abomasum

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One of the most common diseases in high-performance German Holstein dairy cows is left-sided displacement of the abomasum (LDA). Hypomotility of the abomasum is detrimental during the pathogenesis of LDA. It is known that improper interactions between the gut microbiota and the enteric nervous system contribute to dysfunctions of gastrointestinal motility. Therefore, we hypothesized that the gut microbial composition will be different between German Holstein dairy cows with and without LDA. We used 16S rRNA gene analysis to evaluate whether there are any differences in bacterial composition between German Holstein dairy cows with and without LDA. Even though our data are limited to being used to correlate compositional changes with corresponding functional aspects in the pathogenesis of LDA, results from this study show that the fecal microbial compositions of German Holstein dairy cows with LDA shifted and were less diverse than those in normal cows. In particular, *Spirochaetes* were absent in cows with LDA.

One of the most common diseases in high-performance dairy cows is left-sided (LDA) or right-sided (RDA) displacement of the abomasum, collectively known as displacement of the abomasum (DA), in which the abomasum bloats and moves from its normal position on the right ventral part of the abdomen to the left or right abdominal wall (1, 2). DA requires veterinary intervention, and the typical clinical findings of this condition include anorexia and decreased milk production, as DA often occurs near parturition or during the early lactation period (2, 3). Therefore, DA imposes a huge economic burden on dairy producers.

Gas accumulation in the abomasum plays a critical role in the pathogenesis of DA by increasing the buoyancy of the abomasum, resulting in DA. Hypomotility of the abomasum and increased abomasal gas production have been identified as the main mechanisms of action for gas accumulation in the abomasum (1, 3, 4). In a normal situation, the gas in the abomasum is expelled in the oral direction. However, abomasal gas accumulation can occur with hypomotility of the abomasum. Decreased motility of the abomasum is attributed to a large quantity of volatile fatty acids (VFA) in the abomasum, metabolic alkalosis, and low blood calcium levels (1, 4–6). However, abomasal motility is mainly controlled by the vagus nerve, and dysfunction in the vagus nerve can decrease the motility of the abomasum (3, 7–9). It has been shown that the interactions between the enteric nervous system and the gut microbiota in mice contribute to proper gastrointestinal motility (10). The mouse gut microbiota can stimulate vagal sensory neurons, which is a major neural pathway that conveys information from the gastrointestinal luminal contents to the brain, thus modulating gastrointestinal motility (11, 12). In addition, it has been shown that systemic endotoxemia related to the gut microbiota can induce changes in neuronal function, including vagal afferent neurons (13). Therefore, we hypothesized that the gut microbial composition will be different between German Holstein dairy cows with and without LDA. We compared

the fecal microbiota between German Holstein dairy cows with and without LDA.

A total of 20 German Holstein dairy cows at one dairy farm in Chungnam, South Korea, including 8 cows without LDA (control group) and 12 cows with LDA (LDA group), were enrolled in this study (Table 1). All cows involved in this study were at early stage of lactation, and average lactation number was similar between groups, 3.88 ± 1.13 and 8.89 ± 0.93 for the control and LDA groups, respectively.

Animals were housed under the same conditions and were fed the same feed without any antibiotics or supplementary additives. The feed was composed of timothy grass (4.1%), *Festuca arundinacea* (19.1%), oat grass (19.1%), alfalfa bale (6.9%), corn silage (15.3%), and concentrate (35.5%). The nutritive characteristics of concentrate are as follows: crude protein (13.0%), crude fat (10.0%), crude fiber (5.0%), ash (10.0%), calcium (0.4%), phosphorus (0.8%), and total digestible nutrients (90%). LDA was diagnosed by an experienced large-animal veterinarian based on the presence of the characteristic ping on simultaneous auscultation and percussion of the left abdomen and clinical findings, such as anorexia and decreased milk production. Once LDA was diagnosed, fecal samples were collected immediately from the rectum

Received 8 September 2015 Returned for modification 23 October 2015

Accepted 22 January 2016

Accepted manuscript posted online 3 February 2016

Citation Song E-S, Jung SI, Park H-J, Seo K-W, Son J-H, Hong S, Shim M, Kim HB, Song K-H. 2016. Comparison of fecal microbiota between German Holstein dairy cows with and without left-sided displacement of the abomasum. J Clin Microbiol 54:1140–1143. doi:10.1128/JCM.02442-15.

Editor: B. W. Fenwick

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.02442-15>.

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TABLE 1 Animals enrolled in the study

Groups	No. of cows	No. of parturitions ^a	Age (yr) ^a
Control	8	3.88 ± 1.13	5.75 ± 1.49
LDA	12	3.89 ± 0.93	6.56 ± 1.33

^a Values are means ± standard deviations.

and were then frozen at -80°C . Total DNA representing the fecal microbial communities was extracted from individual fecal samples using a stool DNA extraction kit (Bioneer Inc., Seoul, South Korea) according to the manufacturer's recommendations. The 16S universal primers 27F (5' GAGTTTGATCMTGGCTCAG 3') and 800R (5' TACCAGGTATCTAATCC 3') were used to amplify 16S rRNA genes (hypervariable regions V1 to V4). The amplification mix contained 2.5 units of FastStart high-fidelity polymerase (Roche, Mannheim, Germany), 1× FastStart high-fidelity reaction buffer, 0.2 mM concentrations of deoxynucleoside triphosphates (dNTPs), a 0.4 μM concentration of each fusion primer, and 20 ng of DNA in a reaction volume of 50 μL . PCR conditions were an initial denaturation at 94°C for 3 min, 35 cycles of 94°C 15 s, 55°C for 45 s, and 72°C for 1 min, and a final 8-min extension at 72°C . Sequencing and data analysis were conducted as described in our previous studies (14, 15). Briefly, 16S rRNA gene amplicon sequencing targeting regions V1 to V4 were conducted at Macrogen Inc. (Seoul, South Korea) on the Roche 454 GS-FLX sequencer by employing Titanium chemistry (454 Life Sciences, CT, USA) to characterize the fecal bacterial communities in cows. Diversity indices were calculated with an operational taxonomic unit (OTU) definition at an identify cutoff of 97% using mothur, and phylogenetic assessments were performed using the Silva rRNA database and BLASTN (see Table S1 in the supplemental material) (16–18). Statistical analysis was performed using SPSS Statistics 20.0.0 (SPSS Inc., USA). A P value of <0.05 was considered significant. Statistical analyses using the Mann-Whitney U test and the unpaired t test were used to compare microbial compositions and diversity indices between groups, respectively.

We analyzed a total of 186,835 sequence reads after quality control in this study. Average numbers of sequence reads per cow were 7,242 and 10,741 for the control and LDA groups, respectively. Shannon-Weaver and Simpson diversity indices were used to calculate the diversity of microbial communities (see Table S2 in the supplemental material). A higher Shannon-Weaver index indicates a greater sample diversity of microbiota. In the case of Simpson's diversity index, 0 represents infinite diversity while the diversity gradually decreases as the index value becomes close to 1 (18). Decreased microbial diversities have been reported in association with several gastrointestinal disorders (19, 20). As such, the bacterial diversity of the LDA group was significantly lower by the OTU definition at a similarity cutoff of 97% (Table 2). The average Shannon-Weaver and Simpson index values were 4.27 ± 0.16 and 0.02 ± 0.00 for the control group and 3.49 ± 0.63 and 0.08 ± 0.05 for the LDA group. As found with the diversity analysis, the average number of observed OTUs in cows of the control group was also significantly higher than that in the LDA group. The homeostasis of gut bacterial diversity is critical for maintaining gut health (21). As such, our data suggest that lower bacterial diversity is related to the pathogenesis of LDA.

Next, we evaluated whether there are any differences in bac-

TABLE 2 Diversity indices and mean observed OTUs of each group with an OTU definition at an identify cutoff of 97% using mothur

Parameter	Value (mean ± standard deviation) for ^a :	
	Control group	LDA group
Shannon diversity index	4.27 ± 0.16	$3.49 \pm 0.63^*$
Simpson's index	0.02 ± 0.00	$0.08 \pm 0.05^*$
Mean observed OTUs	120.88 ± 35.26	$100.50 \pm 55.64^*$
Total observed OTUs	967	1,206

^a *, $P < 0.05$ between groups.

terial composition between groups. We compared the fecal microbiota between German Holstein dairy cows with and without LDA. The combination of membership and the abundance of each OTU of communities with an OTU definition at an identify cutoff of 97% were compared between groups. A comparison of the community membership and its abundance is critical for evaluation of the degree of differences between microbial communities (22). The composition and relative abundance of each member of the microbiota in feces from the control group were different from those of the LDA group. At the phylum level (Fig. 1A), the fecal bacterial communities of each group were primarily comprised of *Firmicutes* and *Bacteroidetes*, which accounted for more than 95% and 87% of the fecal microbiota, respectively. These results correspond with those of previous studies (23). However, the proportions of these two phyla between groups were not significantly different based on the results of the Mann-Whitney U test ($P > 0.05$). Also, the results of the Mann-Whitney U test showed that the proportion of *Proteobacteria* was not significantly different ($P > 0.05$) between groups. The proportional differences in *Bacteroidetes* and *Proteobacteria* between groups were mainly caused by two outlier cows in the LDA group (Fig. 1A). Prominent differences between the two groups included the presence of *Spirochaetes* in the feces of normal cows (Fig. 1D). The proportion of *Spirochaetes* was significantly different between groups at the phylum level ($P < 0.001$). An average of 1.5% of the microbiota were members of *Spirochaetes* in the feces of the control group. On the other hand, there were no *Spirochaetes* detected in the feces of the LDA group (Fig. 1A).

At the genus level, the relative abundances of five genera were significantly different between groups, while 82.7 and 74.0% of sequence reads were not classified for control and LDA groups (Fig. 1B, C, and D). The proportion of the genus *Enterorhabdus* (a member of the *Actinobacteria*) (Fig. 1B), the proportions of members of the *Firmicutes*, including *Cellulosilyticum*, *Streptococcus*, and *Turicibacter* (Fig. 1C), and the proportion of *Treponema* (a member of the *Spirochaetes*) were all significantly higher in the control group than in the LDA group (Fig. 1D). It is beyond the scope of this study to evaluate the functions of each bacterial group in the pathogenesis of LDA; however, our data suggest that a gut bacterial shift may have detrimental roles during LDA development.

In summary, our data show that the fecal microbial compositions of German Holstein dairy cows with LDA were less diverse than those in control cows. In addition, the relative abundances of genera, including *Enterorhabdus*, *Cellulosilyticum*, *Streptococcus*, *Turicibacter*, and *Treponema*, were significantly higher in the control group than the LDA group. However, current data are limited

FIG 1 The relative abundance of a genus belonging to each phylum. The interquartile range is indicated by the outer bounds of the boxes, the median is indicated by the black midline, and the circles represent the outliers. The whiskers represent the minimum and maximum values. Miscellaneous phyla contained 0.5 and 0.02% of unclassified sequence reads at the phylum level for control and LDA groups, respectively. Control and LDA represent cows without LDA and those with LDA, respectively. (A) Taxonomic analysis of sequences at the phylum level; (B) genus belonging to the *Actinobacteria*; (C) genera belonging to the *Firmicutes*; (D) the phylum *Spirochaetes* and the genus *Treponema*, belonging to the *Spirochaetes*.

to being used to correlate compositional changes with corresponding functional aspects in the pathogenesis of LDA. Therefore, further studies will be needed to elucidate the roles of these genera in the pathogenesis of LDA.

FUNDING INFORMATION

Ministry of Education, Republic of Korea, provided funding to Hyeun Bum Kim under grant number NRF-2015R1D1A1A01061268.

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