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A Low-Starch and High-Fiber Diet Intervention Impacts the Microbial Community of Raw Bovine Milk

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ABSTRACT

Background: A more sustainable dairy cow diet was designed that minimizes use of feed components digestible by monogastric animals by increasing the quantity of forages.

Objectives: This study determined if feeding lactating cows the more sustainable, low-starch and high-fiber (LSHF) diet was associated with changes in raw milk microbiota composition and somatic cell count (SCC).

Methods: In a crossover design, 76 lactating Holstein cows were assigned to an LSHF diet or a high-starch and low-fiber (HSLF) diet, similar to common dairy cow diets in the United States, for 10 wk then placed on the opposite diet for 10 wk. The LSHF diet contained greater quantities of forages, beet pulp, and corn distillers' grain, but contained less canola meal and no high-moisture corn compared with the HSLF diet. Raw milk samples were collected from each cow 4–5 d before intervention and 5 wk into each diet treatment. Within 4 d, additional milk samples were collected for measurement of SCC using Fossmatic 7. The microbial community was determined by sequencing the 16S rRNA gene V4-V5 region and analyzing sequences with QIIME2. After quality filtering, 53 cows remained.

Results: Raw milk microbial communities differed by diet and time. Taxa associated with fiber consumption, such as *Lachnospiraceae*, *Lactobacillus*, *Bacteroides*, and *Methanobrevibacter*, were enriched with the LSHF diet. Meanwhile, taxa associated with mastitis, such as *Pseudomonas*, *Stenotrophomonas*, and *Enterobacteriaceae*, were enriched with the HSLF diet. Relatedly, an interaction of diet and time was found to impact SCC.

Conclusions: In raw milk, consumption of an LSHF diet compared with an HSLF diet was associated with changes in abundance of microbes previously associated with fiber consumption, udder health, and milk spoilage. Further research is needed to determine if an LSHF diet indeed leads to lower rates of mastitis and milk spoilage, which could benefit the dairy industry. *Curr Dev Nutr* 2022;6:nzac086.

Keywords: fiber, starch, microbiota, milk, somatic cell count, cow

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Supplemental Figures 1–5 and Supplemental Tables 1–7 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/cdn/.

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Abbreviations used: DIM, days in milk; DPBS, Dulbecco's phosphate-buffered saline; FDR, false discovery rate; HSLF, high-starch and low-fiber; LSHF, low-starch and high-fiber; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; SCC, somatic cell count; VFA, volatile fatty acid.

Introduction

Agricultural and arable land in the United States, as a percentage of land area, has had a downward trend over the past 2 decades (1, 2). Along with a growing population, this means there is diminishing land available to feed each individual American (3). Yet, more cropland is used to feed livestock, particularly cows, than humans (4). Modifying dairy cow diets to include more forages and fewer crops consumable by monogastric animals, such as corn, could free up croplands and foods for humans and make the cattle industry more sustainable in terms of improving food security (5). In addition, more corn could be available for biofuel production (6).

However, it is not well understood how increasing the forage content in the diet, with simultaneous increase in fiber and decrease in starch, could impact udder health and the bovine microbial community. Previous studies have indicated that high starch content in dairy cattle feed can lead to subacute ruminal acidosis, which is associated with decreased microbial diversity in the rumen, decreased abundance of

cellulose-degrading bacteria, and increased abundance of acid-tolerant bacteria (e.g., *Lactobacillus*), relative to cows given high-fiber feed (7–9). High-starch diets are also associated with greater *Escherichia coli* abundance in cattle feces (10). The microbial content of the digestive system could impact the environmental microbes, which are a major source of contamination for the cow udder and milk (11). On the other hand, there is limited evidence of an entero-mammary pathway, which is hypothesized to transport gut microbiota from the intestines to the mammary glands via mesenteric lymph nodes (12).

In mammals, diet can also affect the nutrients present in the milk, which might indirectly influence the bacterial composition within the mammary tissue based on metabolic needs of various commensal organisms (13–18). There is evidence suggesting that when cows are fed a high concentrate-to-forage diet (i.e., more starch and less fiber) the raw milk microbiota becomes enriched with mastitis-associated bacteria (e.g., *Stenotrophomonas* and *Pseudomonas*) (19) and contains more somatic cells (20). Mastitis is the leading cause of morbidity in dairy cattle and a major economic burden on dairy farmers (21). In 2013, nearly 1 in 4 cows (24.8%) in the United States developed clinical mastitis (22). Some mastitis-associated bacteria, such as *Pseudomonas*, also cause milk spoilage (23, 24). In fact, previous research has found that consumption of a high-starch and low-fiber diet used to induce subacute ruminal acidosis is associated with greater abundance of spoilage bacteria in milk, including *Pseudomonas* and *Enterobacter* (19). Milk and milk products are a major source of food waste, with ∼20% lost for reasons including production losses, processing and packaging losses, consumer losses, and spoilage (25, 26). Therefore, it is critical to consider the potential relation between cattle feed and milk microbiome composition because the latter could affect udder health, milk quality, and milk waste. The primary objective of the feed intervention trial was to measure feed efficiency of cows on a low-starch and high-fiber (LSHF) diet compared with cows on a high-starch and low-fiber (HSLF) diet, while a secondary objective and the focus of this article was to measure differences in milk microbiota and udder inflammation [via somatic cell counts (SCCs)] between cows fed the LSHF diet compared with the HSLF diet. We hypothesized that increasing the fiber quantity and decreasing the starch quantity of dairy cattle feed would lead to alterations in the raw milk microbial community and reduce udder inflammation. To our knowledge, this study is the first to characterize the impact of an LSHF fiber diet on the raw milk microbial community and udder inflammation in a crossover intervention design.

Methods

Bovine diet intervention and milk collection

The impact of an LSHF diet compared with an HSLF diet (**Supplemental Table 1**) on the raw milk microbiota and SCC of 76 Holstein cows in Prairie du Sac, Wisconsin was assessed in a 20-wk nonrandomized crossover trial from October 2017 to April 2018. The regional monthly average minimum, average, and maximum temperatures over the duration of the experiment were gathered from Dane County Regional Airport Station in Madison, Wisconsin from Weather Underground (https://www.wunderground.com) (**Supplemental Table 2**). To obtain feed with "high" and "low" quantities of starch and fiber, the LSHF and HSLF diets were designed with varying amounts of alfalfa silage, corn silage, beet pulp, canola meal, and corn distillers' grain. High-moisture corn was included exclusively in the HSLF diet. Ultimately, diets were balanced for protein availability and other essential nutrients. Sample sizes were calculated to measure the primary outcome of this feed intervention trial—feed efficiency (A Fischer, X Dai, K Kalscheur, unpublished results, 2021). Prior to the start of the first intervention, cows were fed for 31 d a diet comprising 50% of the LSHF diet and 50% of the HSLF diet (**Supplemental Figure 1**). Subsequently cows were assigned to either the LSHF or HSLF diet intervention for 70 d to achieve groups with similar parity, dry matter intake, milk production, and body weight. Consequently, there was no difference in days in milk (DIM) (i.e., lactation stage, mean of 121 and median of 119 d; $P > 0.05$) or parity (median of 2; $P > 0.05$) between cows that began the LSHF diet in period 1 compared with the cows that began the HSLF diet in period 1. Cows were then provided with the opposite diet for an additional 70 d. The first 11 d of a diet intervention period constituted the transition phase. Cows were housed in tie stalls (restricting free movement and pasture grazing) at the same facility in Prairie du Sac, Wisconsin and consumed once a day ad libitum feed amounts adjusted daily to allow a maximum of 10% refusals individually, which was determined with refusals measured 2 d prior.

Milk was collected from the entire udder at 04:00 after teats were stripped (3 streams of milk), treated and disinfected with chlorine dioxide–containing Gladiator predip (BouMatic), and towel dried. Milking equipment was disinfected with iodine in water immediately prior to milking. Teats were also treated and disinfected with iodine-containing Udderdine postdip (BouMatic) to help seal and prevent entry of environmental microbiota into the teat post milking. Two cows, 6230 and 5651, were missed on the fifth day of milk collection (March 14, 2018) during the first morning milk collection; so a milk sample was collected from these cows later that day at 10:30. Raw milk was collected for microbial community analysis on 2 consecutive days after 26 d of consumption of the common diet and after 5 wk of consumption of each experimental diet in portions of ∼48 mL each. Two cows, 6233 and 5849, were missed on the first day of milk collection (November 25, 2021) in period 0. Aliquots were stored at −10°C immediately after collection and shipped on dry ice to the USDA-ARS Western Human Nutrition Research Center. Here the samples were thawed, portioned into smaller 10-mL aliquots, and stored at −70°C until DNA extraction. Evaluating the live bacterial community in these frozen milk samples by culturing methods was not performed because the freezing process likely impacted bacterial viability over time (27).

SCC was measured in raw milk samples collected 1–5 d before or after the milk used for microbial community analysis. Because SCC measures were missing for some cows on certain collection days, only the collection days that had SCC measures for all cows and that were taken soon after the milk collection for milk microbial community were included in SCC analysis (Supplemental Figure 1). SCC measurement was performed by VAS (Valley Agricultural Software) and AgSource laboratories using the FOSSOMATIC 7 flow cytometer. This study was conducted at the USDA-ARS US Dairy Forage Research Center Dairy Farm (Prairie du Sac, WI, USA) under protocols approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (Protocol #A005945).

DNA extraction

DNA was isolated from milk following a protocol that was developed based on the method described by Kable et al. (28). A 10-mL aliquot of raw milk was thawed in a water bath at 4°C for 1 h, then centrifuged at 17,000 × *g* and 4°C for 10 min to separate microbial cells from the milk matrix. The layer of fat on the surface of the centrifuged sample was removed with a sterile tongue depressor and the liquid was decanted. The resulting pellet was washed by suspending in molecular-grade Dulbecco's phosphate-buffered saline, pH 7.4 without calcium or magnesium (DPBS; Gibco), centrifuging for an additional 5 min at 20,000 × *g* and 4°C, and decanting the liquid. The washed pellets were flash frozen on dry ice while awaiting DNA extraction. For each extraction batch, 1 blank sample (negative control, comprised of DPBS), and 1 commercial raw milk sample purchased from a local grocery store, sourced from Duivenvoorden Farms (positive control), were centrifuged, decanted, washed, and extracted alongside the study milk samples.

DNA was extracted from bacterial pellets using the ZymoBIOMICS DNA Miniprep Kit according to the manufacturer's instructions for feces and all nonsoil samples with the exception that after being added to bead tubes, samples were shaken in a FastPrep-24 (MP Biomedicals) twice for 1 min with 5 min rest on ice between cycles. DNA concentration and purity was measured using the Nanodrop (Thermo Scientific) and Qubit 3 fluorometer (Invitrogen) and the dsDNA HS assay kit (Thermo Scientific).

There were several milk samples from which especially low concentrations of DNA (<1.9 ng/μL) were obtained after extraction. Consequently, a second round of DNA extraction was performed on another 10-mL aliquot of the same milk sample.

16S rRNA gene amplification

Bacterial DNA extracted from milk samples, negative controls, and positive controls was sent to the Integrated Microbiome Resource at Dalhousie University (Halifax, Nova Scotia, Canada) for PCR, library preparation, and sequencing. There, amplicons of the V4-V5 region of 16S rRNA genes were generated using primers 515FB and 926R and 25 cycles of PCR, and then were cleaned, normalized, and sequenced for 300 paired ends on the Illumina Miseq platform according to the protocol described by Comeau et al. (29).

Microbial community analysis

Demultiplexed reads were obtained from the Integrated Microbiome Resource and subsequently processed and analyzed using QIIME2 (30). Primers were trimmed off forward and reverse reads using cutadapt (31) and an error rate of 0.106 to allow for 2 mismatches. DADA2 (32) was used with a truncation length of 249 for forward reads and a truncation length of 178 for reverse reads to denoise and dereplicate paired-end reads and remove chimeras. Singleton features were removed from the feature table and the representative sequence file using QIIME feature-table filter features and a minimum feature frequency of 2.

Forty-six potential contaminant features were identified and removed from the feature table based on their prevalence in negative controls relative to experimental samples using decontam (**Supplemental Table 3**) (33). For decontam, the method was set to prevalence with a threshold of 0.5 and samples were grouped by DNA extraction batch. Positive controls were not included in the dataset when running decontam.

TABLE 1 DIM and parity of select cows who began the LSHF diet in period 1 compared with those that began the HSLF diet in period 1[1]

Diet in period 1[2]	DIM on November 25, 2017[3,4]	Parity[3,4]
LSHF	122.38 ± 22.64	1.93 ± 1.28
HSLF	122 ± 25.24	2.04 ± 1.27

[1]DIM, days in milk; HSLF, high-starch and low-fiber; LSHF, low-starch and high-fiber.
[2]Diet treatment types during period 1.
[3]The mean and SD are shown.
[4]Only the cows used in the analysis of taxa associated with diet were included in DIM and parity calculations.

Taxonomic assignments of features were performed using SILVA 138 SSURef NR99 database reference sequences and taxonomic classifications that had been preformatted with RESCRIPt (34, 35). One feature (**Supplemental Table 4**) comprised the majority of reads in the milk samples and was identified as an unnamed taxon of the Bacteria domain with the SILVA database. However, alignment of this feature to DNA sequences in NCBI using blastn indicated this feature is 100% identical to *Bos taurus* mitochondria (accession MT576844.1). This feature was therefore removed from analysis using QIIME feature-table filter features. Other features identified as mitochondria, chloroplast, or eukarya by the SILVA database were also removed using QIIME taxa filter table. After these filtering steps, the median reads per sample was 2934 (**Supplemental Table 5**).

Eleven cows gained access to and consumed food other than their assigned experimental diet, and 10 cows were treated with antibiotic at some time during the study and before or during sample collection due to mastitis, pneumonia, injury, or abscess. Samples derived from these cows were removed from all analyses. The sequence counts per sample were compared between diet groups and between periods (the independent variables of interest). Due to significant differences in median sequence count per sample by these independent variables (**Supplemental Figure 2**), samples were rarefied at the 15th percentile (476 sequences per sample) without replacement to limit the number of false positives, as suggested by Weiss et al. (36). Cows without ≥1 sample per period remaining that met the above criteria were removed, and for cows that had >1 sample in a period, the sample with the higher number of reads was chosen such that only 1 sample per period was remaining for each cow. Ultimately, 159 milk microbiota samples were analyzed representing 3 timepoints for 29 cows that began the LSHF diet in period 1 and for 24 cows that began the HSLF diet in period 1. For this filtered subset of cows, there was no difference in DIM or parity between the cows that began the LSHF diet in period 1 and the cows that began the HSLF diet in period 1 (**Table 1**; $P > 0.05$).

Among this rarefied and filtered dataset, weighted UniFrac distances were calculated and permutational multivariate analysis of variance (PERMANOVA) was conducted with 999 permutations, and permutations were constrained to cow (strata) to determine significant associations between β-diversity and diet, period, and period × diet interaction. The family- and genus-level taxa that were present in ≥33.3% of all samples were examined for differential abundance by diet or period using the Friedman test with repeated measures by cow and false discovery rate (FDR) of 1%. Taxa found to be different by diet or period were then analyzed by pairwise Wilcoxon signed rank test and

FDR of 1%. Similarly, significant differences in microbial class abundance between diets or periods were also determined only for classes corresponding to the genera and families found differentially abundant by diet or period. For these select classes, differential abundance by diet or period was determined using the Friedman test followed by pairwise Wilcoxon signed rank test, as described previously. Experimental groupings were unblinded after the results were identified.

SCC analysis

Associations between SCC and taxa were examined by using the same set of rarefied, filtered data and SCC data from the same cow during the same period. Based on SCC tertiles, samples were divided into "low" (6000–30,000 SCC/mL), "medium" (30,000–96,667 SCC/mL), and "high" (96,667–3,381,000 SCC/mL) SCC groups and compared for differential abundance of family- or genus-level taxa that were significantly different between LSHF and HSLF diets or different between periods 1 and 2. The Wilcoxon rank sum test and FDR of 5% was used to determine which taxa were differentially abundant between the low and high SCC groups.

Associations between diet, period, and SCC were examined using SCC measurements from cows that ate only their designated food, did not receive antibiotics during the study, and had an SCC measurement from each period. SCC were $\log_{10}$-transformed to obtain a normal distribution, and a linear mixed effects model (with cow as the random effect) was fitted and analyzed by ANOVA for the impact of diet, period, and diet × period interaction on SCC.

Results

Associations between raw milk microbiota, period, and diet

Principal coordinate analysis (PCoA) of weighted UniFrac distances for samples from periods 1 and 2 displayed little separation of samples by period or diet (**Figure 1**A). PERMANOVA indicated that there were significant associations between sample β-diversity and period and between β-diversity and period × diet interaction, therefore PCoA was also conducted with centroids for each combination of period and diet (Figure 1B).

The lactation cycle profoundly affects milk yield and composition (37). Therefore, given the longitudinal nature of the crossover study design, we first examined the differential abundance of bacterial taxa in raw milk across intervention periods (period 0, period 1, and period 2), which encapsulated both lactation stage and season. Several genus- and family-level taxa belonging to the classes *Alphaproteobacteria*, *Bacilli*, *Bacteroidia*, and *Gammaproteobacteria* were differentially abundant between periods 1 and 2 (**Supplemental Figure 3**). The differences in relative abundance were significant at the class level for these and *Methanobacteria*, with *Alphaproteobacteria*, *Bacteroidia*, and *Methanobacteria* higher in period 1 than 2, and *Bacilli* and *Gammaproteobacteria* lower in period 1 compared with 2. Notably, *Lactobacillus* was higher in period 1 than 2, whereas *Enterococcus*, *Stenotrophomonas*, and *Enterobacteriaceae* were lower in period 1 than 2 (Supplemental Figure 3). Several of the taxa differentially abundant by period were also differentially abundant by diet.

When we examined the differential abundance of microbial genera and families between LSHF and HSLF diets in raw milk, with correction for cow, we found 11 genera and 14 families differentially abundant (**Figures 2** and **3**). Notably, 3 of these genera—*Bacteroides*, *Pseudomonas*, and an unknown genus of *Lachnospiraceae*—and 5 of these families—*Bacteroidaceae*, *Carnobacteriaceae*, *Christensenellaceae*, *Micrococcaceae*, and *Pseudomonadaceae*—were not differentially abundant between periods 1 and 2. The genera enriched with the LSHF diet included *Lactobacilllus*, *Bacteroides*, and *Methanobrevibacter* (Figure 2). All of these genera, except an unidentified genus of *Lachnospiraceae*, were also enriched on the LSHF diet at the family level (Figure 3). In general, taxa enriched with the LSHF diet belonged to phyla *Firmicutes* and *Bacteroidetes*, particularly within classes *Clostridia* and *Bacteroidia*. These include the families *Sphingobacteriaceae* and *Christensenellaceae*, which were enriched on the LSHF diet at the family level only.

On the other hand, several taxa belonging to the classes *Gammaproteobacteria* and *Actinobacteria* were enriched with the HSLF diet. These included *Pseudomonas*, *Stenotrophomonas*, *Enterobacteriaceae*, and *Bifidobacterium*. However, the mean abundances of *Bifidobacterium* and *Bifidobacteriaceae* did not mirror the direction of significant difference in Wilcoxon signed rank test results therefore we did not display them in the heatmap. At the class level, *Gammaproteobacteria* was significantly higher with the HSLF diet. Notably too, the families *Micrococcaceae* and *Carnobacteriaceae* were enriched on the HSLF at the family level only (Figure 3). At the family and genus levels, there were more taxa differentially abundant between periods 1 and 2 than there were between LSHF and HSLF diets (23 compared with 14 families, and 24 compared with 11 genera, respectively). However, differences in bacterial taxa between diets were robust given that we accounted for period by including cow as a blocking factor in the differential abundance analysis between diets.

Associations between SCC and diet and period

We did not find a difference in the SCC in raw milk from cows on different diets (**Supplemental Figure 4**A) or from cows in different periods (Supplemental Figure 4B). However, there was a significant interaction effect of diet and period on SCC (ANOVA; $P < 0.05$). Although the post hoc pairwise comparisons did not reach statistical significance ($P > 0.05$), for cows that started the LSHF diet in period 1, the raw milk SCC tended to be lower, even after transfer to the HSLF diet, than the raw milk SCC from cows that started the HSLF diet in period 1 (**Figure 4**). Similarly, the change from baseline (period 0) raw milk SCC tended to be negative for cows that began the LSHF diet in period 1 and tended to be positive for cows that began the HSLF diet in period 1 (**Supplemental Figure 5**).

Associations between SCC and raw milk microbiota

After multiple test correction, no diet- or period-associated taxa significantly differed between low and high tertile SCC groups. But prior to multiple test correction, there were 4 family-level taxa—*Oscillospiraceae*, *Bacteroidaceae*, *Muribaculaceae*, and *Bifidobacteriaceae* (**Supplemental Table 6**)—and 5 genus-level taxa—*Bacteroides*, *Kocuria*, an unknown genus of *Muribaculaceae*, *Bifidobacterium*, and an unknown genus of *Oscillospiraceae* (**Supplemental Table 7**)—differentially abundant (Wilcoxon rank sum test $P < 0.05$) between low and high SCC groups. All of these taxa were more abundant in the low SCC group compared with the high SCC group. Of these taxa, *Bacteroidaceae*, *Muribaculaceae*, *Bacteroides,* and an unknown genus of *Muribaculaceae* were also higher in the LSHF diet compared with

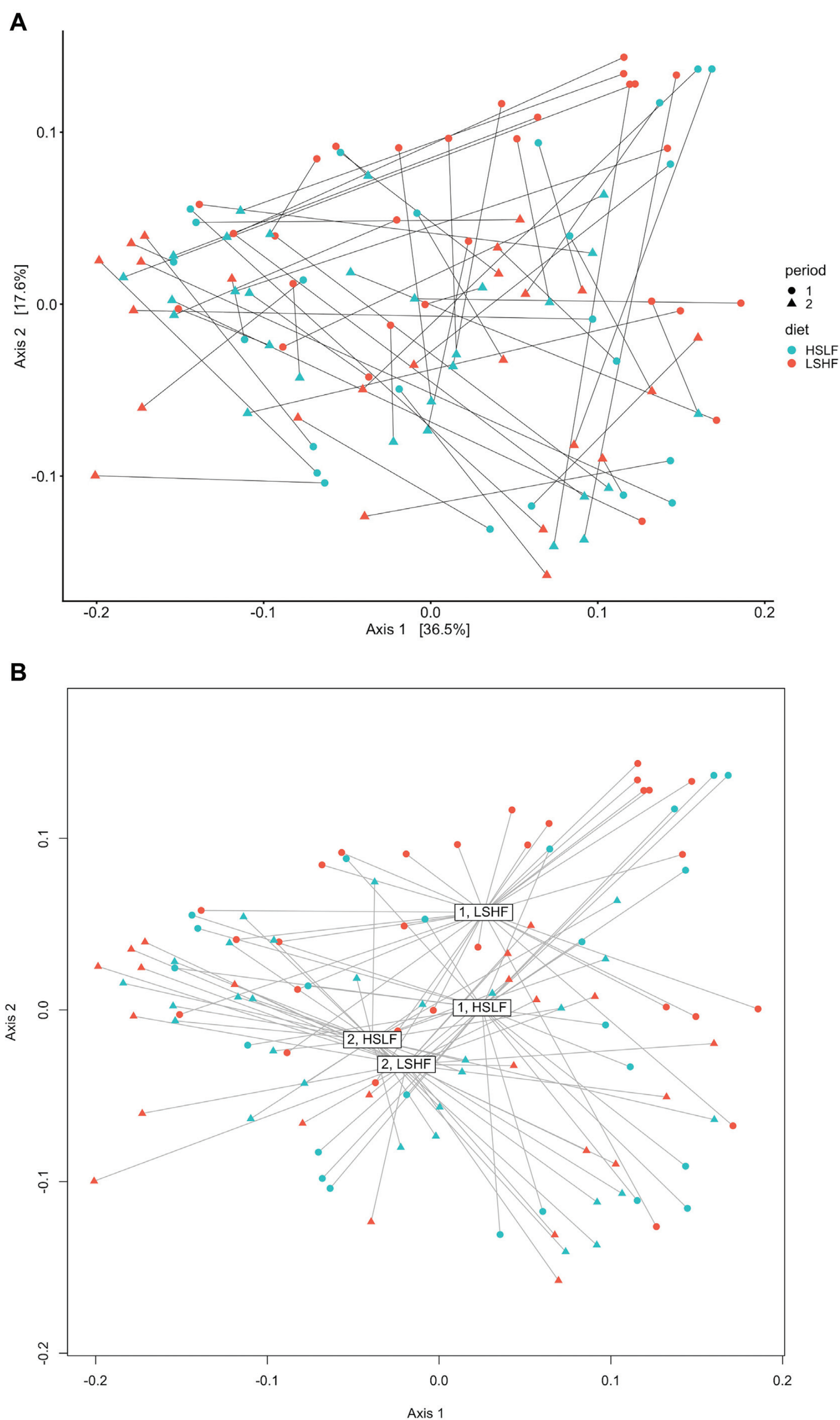

FIGURE 1 Principal coordinate analysis of weighted UniFrac distances for samples during periods 1 and 2. Samples are assigned shapes and colors according to period and diet, respectively. (A) Samples from the same cow are connected by a black line, or (B) samples are connected to the centroids for the diet and period group to which they belong. HSLF, high-starch and low-fiber diet; LSHF, low-starch and high-fiber diet.

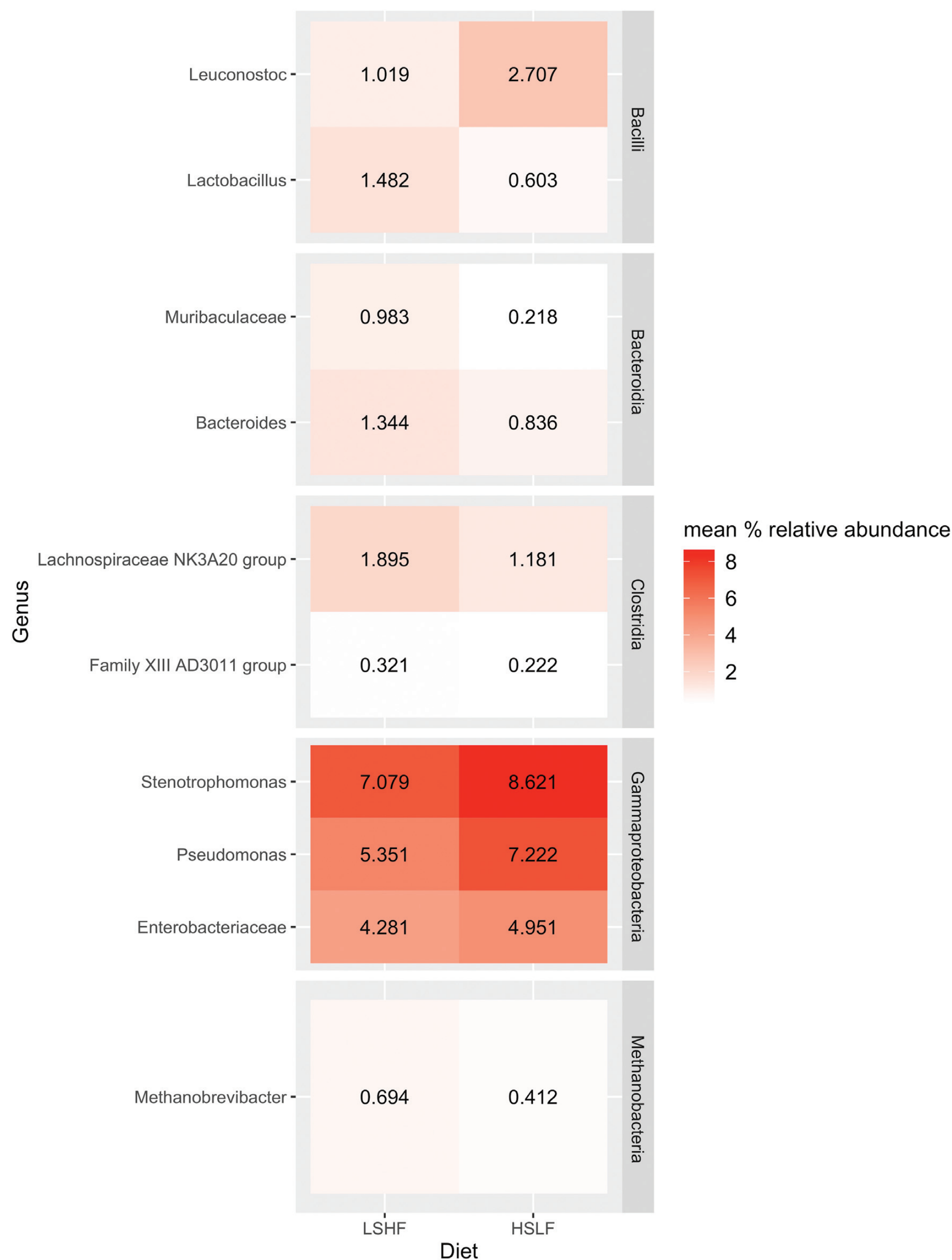

FIGURE 2 Heatmap of the mean relative abundance of rarefied counts for each genus found to be significantly different between the raw milk samples when cows were fed the LSHF diet compared with the HSLF diet. Taxa are organized by class. The genus labeled Family XIII AD3011 group belongs to the *Anaerovoracaceae* family. HSLF, high-starch and low-fiber; LSHF, low-starch and high-fiber.

the HSLF diet (Supplemental Tables 6 and 7). But *Bifidobacteriaceae* and *Bifidobacterium* were higher in the HSLF diet. Also among these taxa, *Oscillospiraceae*, *Muribaculaceae*, *Bifidobacteriaceae*, *Kocuria*, an unknown genus of *Muribaculaceae*, *Bifidobacterium*, and unknown genus of *Oscillospiraceae* were lower in period 1 compared with period 2 (Supplemental Tables 6 and 7).

Discussion

We found that increasing fiber content of cattle feed was associated with an enrichment of bacterial taxa in milk that are capable of degrading fiber, and also associated with a depletion of *Gammaproteobacteria* and various mastitis-associated families and genera within

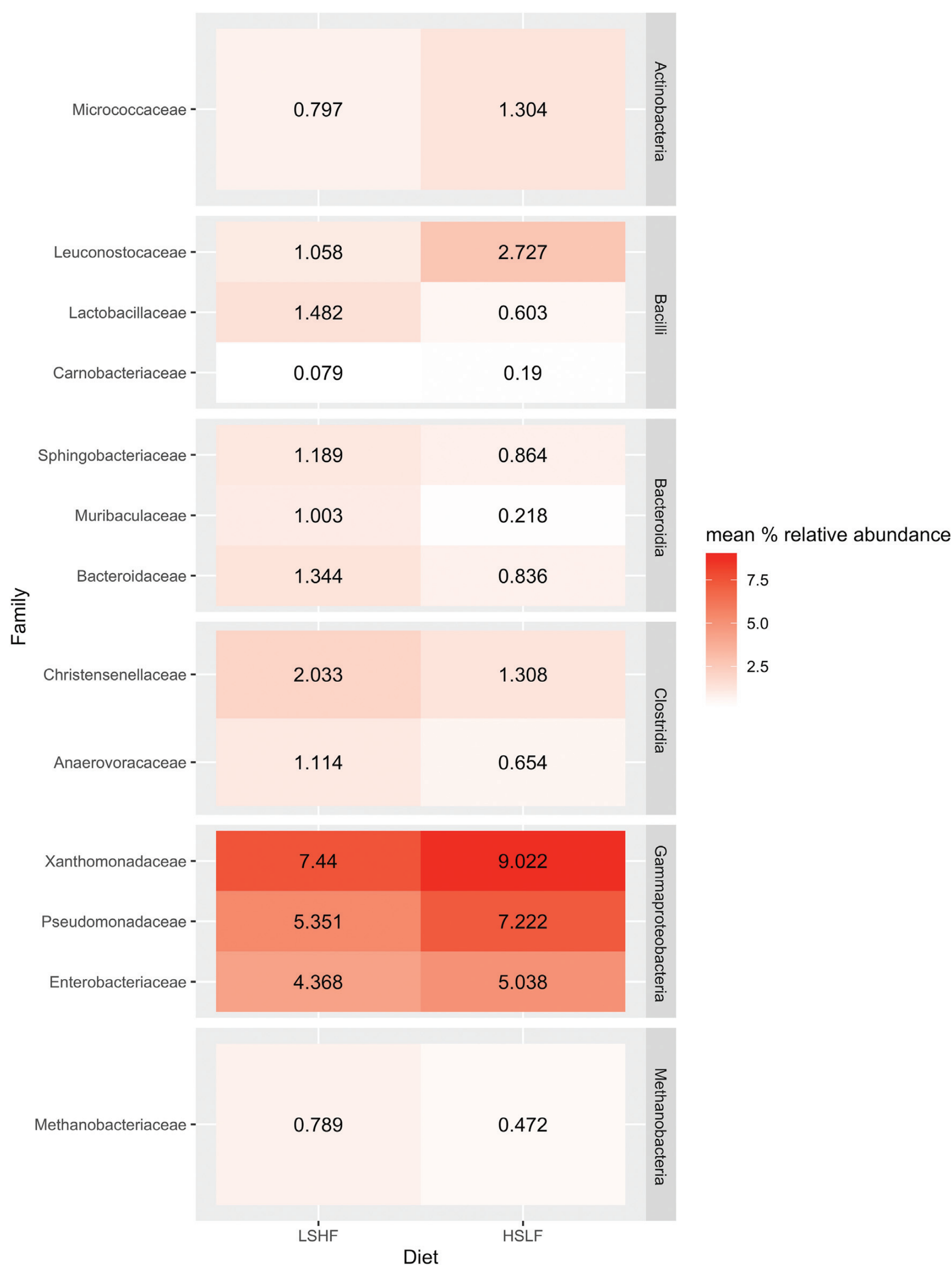

FIGURE 3 Heatmap of the mean relative abundance of rarefied counts for each family found to be significantly different between the raw milk samples when cows were fed the LSHF diet compared with the HSLF diet. Taxa are organized by class. HSLF, high-starch and low-fiber; LSHF, low-starch and high-fiber.

Gammaproteobacteria. We also found a significant interaction effect of diet and period on SCC and a pattern for decreased SCC over time when the LSHF diet was started earlier in lactation, suggesting that high-fiber cattle feed should be investigated further in relation to prevention of mastitis.

Our examination of the microbial communities in raw milk showed that feeding an LSHF diet compared with an HSLF diet was associated with an enrichment of *Lactobacillus*, *Bacteroides*, *Muribaculaceae*, an unknown genus of *Lachnospiraceae*, and *Methanobrevibacter*, which can ferment complex oligosaccharides or are associated with high fiber consumption in the mammalian gut (38–42). Unexpectedly though, *Bifidobacterium*, which can also ferment complex oligosaccharides and is associated with high fiber consumption in mammals (15, 38, 39), was enriched with the HSLF diet. *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* are beneficial, health-promoting bacteria for cows (43, 44) and are associated with healthy udders compared with mastitic udders (45, 46). *Bifidobacterium* and *Bacteroides* also tended to be higher in milk samples from cows that also had low SCCs. Previously,

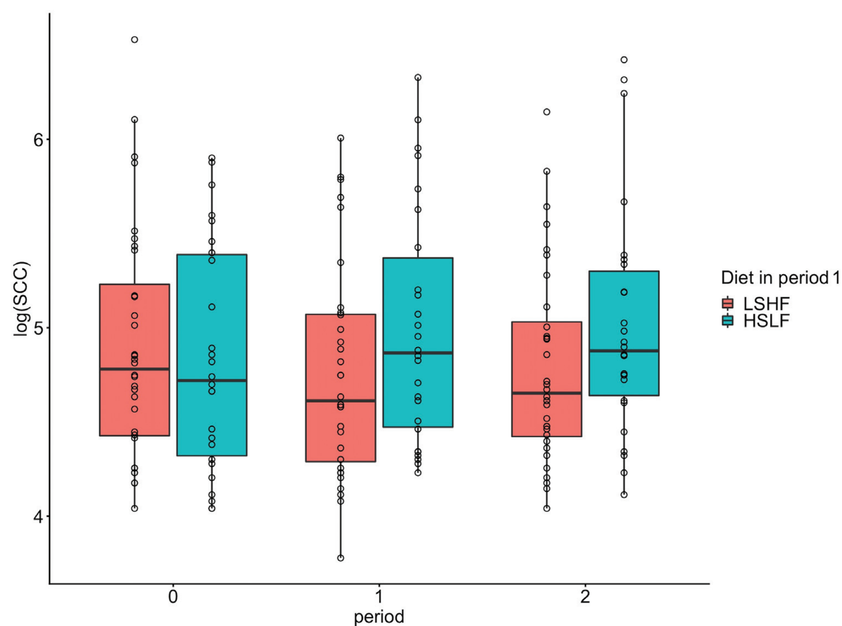

FIGURE 4 The log-transformed SCC by period with distinction of diet order. Boxplots of log(SCC) from cows that began the LSHF diet in period 1 are colored red whereas boxplots of log(SCC) from cows that began the HSLF diet in period 1 are colored blue. Boxplots display first, second, and third quartiles, and the smallest and largest values at most 1.5 times the IQR. HSLF, high-starch and low-fiber; LSHF, low-starch and high-fiber; SCC, somatic cell count.

Bifidobacterium and *Lactobacillus* were used to effectively treat mastitis, particularly through oral delivery (47, 48). The high abundance of *Lactobacillus* and *Bacteroides* might explain the reduced abundance of the mastitis-associated bacteria *Stenotrophomonas*, *Pseudomonas*, and *Enterobacteriaceae* that we observed with the LSHF diet (23, 49–51). Importantly too, *Pseudomonas* and numerous strains within *Enterobacteriaceae* are psychrotrophic bacteria, which can lead to milk spoilage, so the resulting lower abundances in the LSHF diet could have a positive impact on spoilage rates of milk (24, 52).

The mechanisms governing the relation between cattle feed and milk microbiota remain unclear and were outside of the scope of this study. Previous research suggests bedding and feces are a source of bacteria found in udders (53). However, we did not have fecal samples from the cows to determine if there was a relation between the fecal microbiota and the milk microbiota. It is also worthwhile to consider that the HSLF diet could have indirectly impacted the milk microbiota by causing an increase in volatile fatty acids (VFAs) in the rumen that outpaces ruminal epithelial absorption of VFAs and consequently lowers ruminal pH (54). Abnormally low pH can disrupt the rumen epithelial barrier and permit more LPSs to enter the bloodstream. If abundant enough in the bloodstream, LPSs can overwhelm the liver and reach the mammary tissue. LPSs in the mammary glands increase permeability of the mammary epithelium and allow more neutrophils to enter the mammary glands. Consequently, inflammation and susceptibility to mastitis increase (12). Inflammation causes increased oxygen along the epithelium, which allows facultative anaerobes, such as *Gammaproteobacteria*, to proliferate (55, 56). This mechanism might explain why the HSLF diet was associated with greater abundance of mastitis-associated *Gammaproteobacteria* in the raw milk.

Diet can also indirectly modify the milk microbial community by changing the nutritional composition of milk. In cows, high-fiber diets are associated with greater milk fat content (13), which in turn is associated with higher milk microbiota richness (14). Others analyzed the milk fatty acids and the milk oligosaccharides from the same cows from which we collected samples and found significant associations between diet and milk fatty acid composition (57) and between diet and milk oligosaccharide composition (58). The quantity of fiber consumed is also associated with milk oligosaccharide composition in rats (15). In humans, milk oligosaccharides are prebiotics for commensal bacteria, such as *Bifidobacterium*, *Bacteroides*, and *Lactobacillus*, and can also serve as decoys for pathogens to prevent attachment to host cells (11, 59–61). Milk oligosaccharides from other mammals likely provide similar protective functions to the neonate as well (17). The correlation of certain human milk oligosaccharides with milk microbiota suggests that oligosaccharides can also impact the milk microbial community (18). Future work should determine if particular bovine milk oligosaccharides correlate with *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* abundances or mastitis-associated bacteria.

Beside diet, there are multiple factors that affect the milk microbial community and the risk of developing mastitis. These include lactation stage, parity, and season (11, 59–61). Thus, we also hypothesized that, as cows consumed the experimental diets over several months, lactation stage and season (i.e., period) would have an effect on milk microbial community and udder inflammation as well. We found that the milk microbial composition differed by period and the period × diet interaction, which suggests that period can have a greater impact on the milk microbiota than the LSHF diet compared with the

HSLF, and that lactation stage and season are likely important factors in diet intervention for cows. Furthermore, we found numerous taxa differentially abundant between periods 1 and 2. Some of the taxa that we found to be associated with period in our study have also been associated with season in earlier research, including *Prevotellaceae*, *Sphingobacteriaceae*, *Planococcaceae*, *Enterococcaceae* (62), *Microbacteriaceae* (63), *Lactobacillus*, *Stenotrophomonas*, *Bifidobacterium*, *Chryseobacterium*, and *Enterococcus* (64). Likewise, others have found associations between lactation stage and milk microbiota for cows and humans, including associations between *Chryseobacterium* abundance and lactation stage in cows (65), and *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* abundances and lactation stage in humans (66), as we found for period in our study. However, substantial differences in experimental design exist between our study and these previous studies, such as cow breed, country, time of year, lactation stage, and cow environment, so that differences in findings are not unexpected. Certain taxa, such as the commensal *Lactobacillus*, decreased with time whereas mastitis-associated bacteria—*Enterococcus* (67), *Stenotrophomonas*, and *Enterobacteriaceae*—increased with time in our study. These changes in the microbial community could relate to previously reported increases in SCC from mid to late lactation—similar to the lactation stages captured in this study.

Although we found that more taxa were differentially abundant between periods 1 and 2 than between the LSHF and HSLF diets, we accounted for this effect by blocking for cow when comparing the abundance of bacteria between diets and we found several genera and families that were only differentially abundant between the LSHF and the HSLF diets. These included *Bacteroides*, *Pseudomonas*, and an unknown genus of *Lachnospiraceae* as well as *Bacteroidaceae*, *Carnobacteriaceae*, *Christensenellaceae*, *Micrococcaceae*, and *Pseudomonadaceae*. As mentioned previously, *Bacteroides* is associated with healthy udders whereas *Pseudomonas* is associated with mastitis and spoilage. Consequently, an LSHF diet could have impacted the abundance of certain milk microbial taxa, irrespective of lactation stage or season, in ways that potentially could have positive implications for mastitis and spoilage risk.

Meanwhile *Bifidobacterium*, *Lactobacillus*, *Stenotrophomonas*, and *Enterobacteriaceae* were some of the taxa that differed by diet and by period. These bacteria are associated with mastitis, but it is unknown if their abundance impacts the risk of developing mastitis. This result combined with our finding that the interaction of period with diet had a significant effect on SCC indicates that future research should consider lactation stage and season when characterizing the relation between diet, mammary inflammation, and mastitis.

The results presented here are associations that do not necessarily demonstrate cause-and-effect relations between diet and inflammation in the udder. The conclusions that can be reasoned from the findings in this study are somewhat limited because only a single timepoint per period was examined, which did not provide a comprehensive measure of how the milk microbial community and milk SCC changed with diet over the course of each intervention period, and the examination of frozen milk samples prevented the differentiation between live and dead microbes at the time of collection. Although disinfectants were used to limit introduction of microbes from the outer teat surface and milking equipment into the raw milk samples, we could not be certain that the microbes that we did detect in the raw milk originated from within the udders. Notably too, the observed relations between diet and milk microbiota and SCC are associative, not causal, therefore it is possible there are variables we did not measure that better explain the changes in milk microbiota. Still, our study results serve as preliminary evidence for a hypothesis relating cow diet to milk microbial composition and udder inflammation.

In sum, our work provides preliminary evidence suggesting that a sustainable high-forage and high-fiber cattle feed is associated with a healthy milk microbiota composition and lower SCC over time. An LSHF diet was associated with increased abundance of commensal microbes and reduced the abundance of mastitis-associated and spoilage-causing bacteria relative to an HSLF diet. More work is necessary to confirm the relations between low starch and high fiber consumption, milk microbiota, and udder inflammation and to determine if the changes in milk microbiota or udder inflammation ultimately impact rates of mastitis, spoilage, and waste.

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