J. Dairy Sci. 104:4119–4133
https://doi.org/10.3168/jds.2020-19291

Evaluating the effects of *Lactobacillus animalis* and *Propionibacterium freudenreichii* on performance and rumen and fecal measures in lactating dairy cows

M. Lawrence,[1] S. Polukis,[1] A. M. Barnard,[1] M. A. Miller,[2] L. Kung Jr.,[1] and T. F. Gressley[1]*
[1]Department of Animal and Food Sciences, University of Delaware, Newark 19716
[2]Phaseolus Consulting, Wyndmoor, PA 19308

ABSTRACT

Two experiments evaluated the effect of supplementation with a bacterial direct-fed microbial on performance and apparent total-tract nutrient digestion of dairy cows. In experiment 1, 30 multiparous cows (75 ± 32 d in milk) were randomly assigned to 1 of 2 treatments fed for 10 wk. All cows were fed a diet containing 23.8% starch. Treatments were top dressed to rations twice daily and consisted of a combination of *Lactobacillus animalis* (1×10^9 cfu/d) and *Propionibacterium freudenreichii* (2×10^9 cfu/d; LAPF) or carrier alone (CON). In experiment 2, 6 ruminally cannulated cows (123 ± 129 d in milk) were randomly assigned to a crossover design with two 6-wk periods. Cows received the same CON or LAPF treatment as in experiment 1. Cows were fed the same 23.8% starch diet as experiment 1 during wk 1 through 5 of each period, and then cows were abruptly switched to a 31.1% starch diet for wk 6. For both experiments, intake and milk yield were measured daily, and milk samples were collected weekly. In experiment 1, fecal grab samples were collected every 6 h on d 7 of experimental wk 1, 2, 4, 6, 8, and 10. Fecal consistency was scored, and fecal starch was measured in daily composite samples. Fecal composites from a subset of 7 cows per treatment were used to measure apparent total-tract nutrient digestion. In experiment 2, rumen pH was continuously recorded during wk 5 and 6. On d 7 of wk 5 (the final day of feeding the 23.8% starch ration), d 1 of wk 6 (the day of diet transition), and d 7 of wk 6 (the final day of feeding the 31.1% starch ration), rumen in situ digestion was determined. Samples of rumen fluid and feces were collected every 6 h on those days for measurement of fecal starch (composited by cow within day), rumen volatile fatty acids, and fecal pH. Rumen and fecal samples were collected at one time point on those days for microbiota assessment. In experiment 1, treatment did not affect intake, milk yield, milk composition, or fecal score. The LAPF treatment decreased fecal starch percentage and tended to increase starch digestion compared with CON, but the differences were very small (0.59 vs. 0.78% and 98.74 vs. 98.46%, respectively). Digestion of other nutrients was unaffected. In experiment 2, LAPF increased rumen pH following the abrupt switch to the high-starch diet, but milk yield was lower for LAPF compared with CON (35.7 vs. 33.2 kg/d). Contrary to the decrease in fecal starch with LAPF observed in experiment 1, fecal starch tended to be increased by LAPF following the abrupt ration change in experiment 2 (2.97 vs. 2.15%). Few effects of treatment on rumen and fecal microbial populations were detectable. Under the conditions used in our experiments, addition of the bacterial direct-fed microbials did not have a marked effect on animal performance, ruminal measures, or total-tract nutrient digestion.

Key words: direct-fed microbial, total-tract nutrient digestion, starch

INTRODUCTION

Bacterial direct-fed microbials (**DFM**) may improve productivity and feed efficiency of dairy cattle. Potential modes of action for these benefits include modifying ruminal and intestinal microbial populations and fermentation patterns, competitive exclusion of intestinal pathogens, and modifying intestinal permeability and immune function (Krehbiel et al., 2003). Lactic acid-producing bacteria (**LAB**) are among the most commonly supplemented DFM (McAllister et al., 2011). In the rumen, LAB increase lactic acid production, which is thought to help maintain populations of lactic acid-utilizing bacteria (**LUB**) and, thus, make the rumen more stable when challenged with diets containing large quantities of rapidly fermentable carbohydrates (Yoon and Stern, 1995). Additionally, many LAB can arrive intact to the intestine, where they can

Received July 14, 2020.
Accepted November 23, 2020.
*Corresponding author: gressley@udel.edu

exert beneficial effects, most consistently manifested as a decrease in fecal shedding of pathogenic organisms (Wisener et al., 2015). Lactic acid-utilizing bacteria can also be fed as DFM, either alone or in concert with LAB. *Propionibacterium* are LUB that convert lactic acid into propionate, acetate, and CO_2 (Krehbiel et al., 2003), and increased propionate production as a result of feeding LUB can increase productive efficiency (Weiss et al., 2008).

Theoretically, feeding LAB with propionate producing LUB should help to stabilize the rumen environment, prevent rumen acidosis, and increase propionate absorption and productive efficiency. One commercially available LAB and LUB combination contains *Lactobacillus animalis* and *Propionibacterium freudenreichii*. However, responses to this DFM mixture have been variable, with some finding improvements in milk yield or productive efficiency (West and Bernard, 2011; Kenney et al., 2015) but others reporting no difference (Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015). Some of the variability in response may be related to the level of stress that the animals are experiencing, because DFM are likely to be of greatest benefit during times of stress (Seo et al., 2010), and this combination has been successful during periods of heat stress (Boyd et al., 2011). A challenge that dairy cattle may face is feeding inconsistencies due, for example, to mixing error, forage changes, equipment inaccuracy, and changes in weather or storage conditions. Direct-fed microbials that contain or stimulate LUB may help to stabilize the rumen environment during unintentional dietary shifts.

The goal of this work was to evaluate responses of dairy cows to *L. animalis* and *P. freudenreichii* under 2 different conditions. The objective of experiment 1 was to evaluate the effects of the DFM on milk yield, feed intake, and total-tract nutrient digestion in early-lactation cows. We hypothesized that the DFM treatment would increase digestive efficiency, which would manifest as increased nutrient digestion and increased milk yield or productive efficiency. The objective of experiment 2 was to determine the effects of the DFM on rumen pH, rumen VFA, and rumen and fecal microbiota before and following a dietary challenge that consisted of an abrupt ration change. We hypothesized that the DFM treatment would result in a more stable rumen environment following the transition to the higher-starch ration.

MATERIALS AND METHODS

Animals and Treatments

All animal procedures took place at the University of Delaware (Newark) and were approved by the University of Delaware Institutional Animal Care and Use Committee protocol 66R. Experiment 1 was conducted from Nov. 2, 2016, through Jan. 24, 2017, and experiment 2 was conducted from Oct. 27, 2016, through Jan. 24, 2017.

Experiment 1. Thirty multiparous Holstein dairy cows were used in experiment 1. Cows were eligible to be enrolled if they were multiparous, in early lactation, and free of clinical signs of disease. Cows were housed in a 30-cow sand-bedded freestall barn and were fed individually via a Calan gate system (American Calan, Northwood, NH). At the start of the trial, mean (±SD) DIM was 75 ± 32, and milk yield was 49 ± 6 kg/d. Cows were fed once daily at approximately 0800 h for ad libitum intake, and refusals were removed and weighed daily for measurement of daily intake. Cows were milked twice daily at approximately 0430 and 1600 h, with milk weights recorded at each milking. Cows were weighed on 2 consecutive days before the start of the experiment and at the end of wk 5 and 10.

The experiment was conducted over 12 wk, with a 2-wk baseline period followed by a 10-wk experimental period. During the baseline period, all cows were fed a total mixed ration without bacterial DFM (Table 1). At the end of the baseline period, cows were blocked into pairs by milk yield and DIM. Members of each pair were assigned to 1 of 2 treatments according to a randomized block design. Treatments were assigned using a random number generator by author SP. The treatments were control (**CON**) and supplementation with *L. animalis* and *P. freudenreichii* (**LAPF**). During the treatment period, cows on the CON treatment continued to be fed the ration without bacterial DFM, whereas cows on the LAPF treatment received the same ration but supplemented with 28 g/d of a commercial blend of *L. animalis* (1×10^9 cfu/d) and *P. freudenreichii* (2×10^9 cfu/d; Bovamine, Chr. Hansen, Hørsholm, Denmark). The DFM supplement was provided as a twice-daily topdress mixed with a ground corn grain carrier (100 g/feeding, 200 g/d) given at approximately 0800 and 1700 h. The dry DFM supplement was stored in an airtight container in a laboratory at room temperature. The topdress mixture was weighed out weekly into resealable plastic bags that were sealed and stored in a temperature-controlled office at the dairy until feeding times. The time that elapsed between the mixing of the topdress and presentation to the animals varied depending on the day of the week, from 1 to 7 d. Viability of the supplement was not determined. At each of those times, cows on the CON treatment received 100 g of corn grain plus 14 g of a 50/50 mixture of dried distillers grains and calcium carbonate. Cows remained on their respective treatment until the completion of the 10-wk experimental period.

Table 1. Ingredient composition and analyzed nutrient composition (% of DM unless otherwise noted) of the experimental rations

Item	Experiment 1: wk 1–10 Experiment 2: wk 1–5	Experiment 2: wk 6
Ingredient		
Corn silage	51.47	45.61
Alfalfa silage	8.90	7.89
Alfalfa hay	8.58	7.61
Ground corn	8.02	18.47
Protected soybean meal[1]	6.92	6.13
Canola meal	5.42	4.81
Citrus pulp	2.34	2.08
Sugar byproduct[2]	1.67	1.48
Porcine blood meal	1.64	1.45
Rumen bypass fat[3]	1.39	1.23
Sodium bicarbonate	0.73	0.65
Corn gluten meal	0.54	0.48
Trace mineral and vitamin mix[4]	0.46	0.41
Sodium chloride	0.37	0.33
Calcium carbonate	0.32	0.28
Potassium carbonate[5]	0.30	0.27
Monensin[6]	0.29	0.26
Monocalcium phosphate	0.28	0.25
Methionine precursor[7]	0.083	0.076
Potassium and magnesium sulfate[8]	0.061	0.053
Rumen protected methionine[9]	0.053	0.045
Urea	0.049	0.045
Rumen-protected lysine[10]	0.042	0.038
Vitamin E, 46 kIU/kg	0.034	0.030
Magnesium oxide	0.023	0.023
Chelated zinc[11]	0.008	0.008
Biotin[12]	0.004	0.004
Nutrient (±SD)		
DM, %	45.8 ± 2.3	47.8 ± 1.9
CP	16.2 ± 0.2	15.5 ± 0.2
NDF	32.3 ± 1.7	28.6 ± 0.3
ADF	21.8 ± 1.1	19.0 ± 0.1
Starch	23.8 ± 2.1	31.1 ± 0.3
Ash	7.0 ± 0.6	6.8 ± 0.6
NE_L	1.69 ± 0.02	1.72 ± 0.03

[1]Extruded and expelled soybean meal (J. L. Moyer and Sons Inc., Turbotville, PA).

[2]Contained 92.3% sucrose (Renaissance Nutrition Inc., Roaring Spring, PA).

[3]MEGALAC (Church and Dwight Co. Inc., Princeton, NJ).

[4]Contained 14.7% calcium, 34.3% magnesium, 0.75% sulfur, 102 mg/kg Fe, 4,262 mg/kg Zn, 823 mg/kg Cu, 4,215 mg/kg Mn, 65.5 mg/kg Se, 141 mg/kg Co, 191 mg/kg I, 191 mg/kg I, 1,268 kIU/kg vitamin A, 254 kIU/kg vitamin D, and 5,062 IU/kg vitamin E.

[5]DCAD Plus (Church and Dwight Co. Inc.).

[6]Custom premix produced by Renaissance Nutrition, containing 0.485% Rumensin 90 (Elanco, Greenfield, IN).

[7]HMTBa (MFP, Novus International Inc., St. Charles, MO).

[8]Dynamate (18% K, 11% Mg, 22% S; the Mosaic Company, Plymouth, MN).

[9]Smartamine M (Adisseo, Antony, France).

[10]AjiPro-L Generation 2 (Ajinomoto Heartland Inc., Chicago, IL).

[11]Mintrex Zn (Novus International Inc.).

[12]Microvit H Promix Biotin 2% (Adisseo).

Experiment 2. Six lactating (4 multiparous and 2 primiparous) Holstein cows were housed in tiestalls. Cows were fitted with rumen cannulas before the start of the experiment and were eligible to be enrolled if they had rumen cannulas and were free of clinical disease symptoms. Cows had a mean milk yield of 36 ± 15 kg/d and DIM of 123 ± 129. The high variability in milk and DIM was due to need to replace a cow at the start of the experiment, and only a very late-lactation (11 kg/d milk, 377 DIM) rumen-cannulated cow was available. The milk yield and DIM for the other 5 cows was 40 ± 8 kg/d and 73 ± 32, respectively. Body weight (mean ± SD) measured over 2 consecutive days at the start of the trial was 715 ± 32 for the multiparous cows and 590 ± 22 for the primiparous cows, respectively. Cows were fed twice daily (0900 and 1630 h)

for ad libitum intake, and refusals were removed and weighed daily for measurement of daily intake. Cows were milked twice daily (0430 and 1600 h), with milk weights recorded at each milking.

The experiment was conducted as a crossover design with two 6-wk periods. Cows were blocked by parity and assigned to 1 of 2 treatment sequences using a random number generator by author ML. During each 6-wk period, cows received either no bacterial DFM (CON) or the LAPF treatment as described for experiment 1. During the first 5 wk of each period, cows were fed the same ration as in experiment 1, containing 23.8% starch, but during wk 6 cows were abruptly switched to a ration containing 31.1% starch (Table 1).

Experiment 1 Sampling and Analysis

Milk and Feed. Milk samples were collected at both daily milkings one day each week and analyzed by Dairy One (Ithaca, NY). Lactose, protein, fat, and MUN were measured using a MilkoScan System 4000 (Foss, Hillerød, Denmark) and SCC using a Fossomatic 400 (Foss).

Silage and TMR samples were collected 3 times a week, and concentrate and hay samples were collected once each week. A portion of each sample was used for DM determination by drying for 48 h in a forced-air oven at 60°C, with results used for DMI determination and weekly DM adjustments of TMR ingredient amounts. The remainder of each sample was stored at −20°C until compositing at 2-wk intervals. Composite samples were mailed to Cumberland Valley Analytical Services (Waynesboro, PA) for wet chemistry analysis of DM (105°C for 3 h for forages; method 930.15, AOAC International, 2000, for grain), NDF (Van Soest et al., 1991), ADF (method 973.18, AOAC International, 2000), CP (method 990.03, AOAC International, 2000), starch (Hall, 2009), ash (method 942.05, AOAC International, 2000), and minerals (method 985.01, AOAC International, 2000). Net energy for lactation at 3 × maintenance was calculated from nutrient composition using NRC (2001) equations.

Apparent Total-Tract Nutrient Digestion and Fecal Measures. Fecal samples were collected from all cows at the end of the baseline period (wk −1), at 1 and 2 wk following the start of the treatment period, and every 2 wk thereafter (wk 4, 6, 8, 10). On d 7 of each of those weeks, fecal grab samples were collected at 4 time points (0900, 1500, 2100, and 0300 h). Fecal score (1 = liquid to 5 = extremely well formed; Hulsen (2006)) was recorded, and the samples were stored at −20°C until composited into 1 sample per cow per day and dried at 60°C for 48 h. Two independent TMR samples were also collected from the morning feeding on the day of fecal sampling by using a small shovel to collect TMR from 10 different feed bins into a 20-L plastic bucket. Independent samples were sequentially mixed and halved and then frozen until analysis.

Dried daily composite fecal samples from a subset of 14 cows (7 per treatment) were used for measurement of total-tract apparent nutrient digestion. Fecal samples and corresponding TMR samples collected on the same dates were analyzed by Cumberland Valley Analytical Services for CP, NDF, ADF, starch, and ash, as described above. In addition, 240-h undigested NDF was determined in vitro (Goering and Van Soest, 1970). Total-tract apparent digestion of CP, NDF, ADF, starch, and OM were calculated for each of the 14 cows using 240-h undigested NDF as an internal marker. Dried daily composite fecal samples from the remaining 16 cows were analyzed for starch only.

Experiment 2 Sampling and Analysis

Milk and Feed. During each period, milk samples were collected at both milkings on d 7 of each week during wk 1 through 5 and on d 1, 3, and 7 of wk 6. Samples were analyzed for lactose, protein, fat, SCC, and MUN as described for experiment 1. Samples of wet forages were collected 3 times a week and dry feeds collected once weekly. Feed sample composites were generated for wk 1 and 2, 3 and 4, 5, and 6, and analyzed for nutrient composition as described for experiment 1.

Rumen and Fecal Measures. Rumen pH was measured continuously at 5-min intervals during wk 5 and 6 in all cows using indwelling pH meters (T7-1 Data Loggers, Dascor, Escondido, CA). In situ digestion was measured d 7 of wk 5, d 1 of wk 6, and d 7 of wk 6. On those dates, dried and ground TMR from the 23.8% starch ration was placed in Dacron bags (4.0 ± 0.1 g of TMR in 10 × 20-cm bags with 50-µm porosity; Ankom Technology, Macedon, NY) in the rumen and incubated in triplicate in each cow to evaluate DM disappearance after 6, 12, 18, and 24 h in the rumen. Timing of bag placement occurred such that bags were placed in the rumen at different times (4, 10, 16, and 22 h following a.m. feeding), but all bags were removed from the rumen at the same time (4 h following a.m. feeding). A single sealed empty bag was included for each cow and each time point, to correct for weight gain due to rumen incubation alone.

At each time of Dacron bag placement (4, 10, 16, and 22 h relative to the a.m. feeding), rumen fluid and fecal grab samples were collected for measurement of rumen VFA, fecal pH, and fecal starch. Two researchers worked in tandem at each sampling time. One researcher placed Dacron bags and collected and processed rumen fluid samples. The second researcher collected and

processed fecal samples. Rumen fluid was composited from 4 different locations within the ventral rumen sac and strained through 2 layers of cheesecloth. Ten milliliters of rumen fluid was preserved with 0.2 mL of 50% H_2SO_4 and stored at −20°C until analysis of VFA and lactic acid via HPLC (Muck and Dickerson, 1988). For measurement of fecal pH, 20 g of feces was added to 20 mL of water and shaken vigorously for 20 s, and the liquid was squeezed through 4 layers of cheesecloth. The pH of the liquid was then measured using a portable pH meter (P771, Anaheim Scientific, Yorba Linda, CA). Fecal samples for starch determination were stored at −20°C until composited by cow within each sampling day and dried for 48 h at 60°C. Starch content of fecal composite samples was analyzed by Cumberland Valley Analytical Services as previously described.

Rumen and Fecal Microbiota Analysis. Samples of rumen fluid and feces collected 10 h after the a.m. feeding on d 7 of wk 5, d 1 of wk 6, and d 7 of wk 6 were also used for microbiota analysis. All equipment used for rumen fluid and feces sampling was sterilized before use. Rumen fluid and feces samples were stored at −80°C in 5-mL cryovials until being shipped on dry ice to RTL Genomics (Lubbock, TX) for sequencing.

Extraction of DNA, library preparation, amplification, and sequencing were performed by RTL Genomics. Extraction of DNA used the PowerMag Soil Kit according to manufacturer instructions (MO BIO Laboratories Inc., Carlsbad, CA). The primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) were used to amplify the V4 and V5 hypervariable regions of the 16S rRNA gene. Samples were amplified for sequencing using the Illumina 2-step process. The Illumina MiSeq (San Diego, CA) platform with the 250-bp paired-end method was used for sequencing.

Sequence data were analyzed using QIIME 2 (Bolyen et al., 2019). Sequences were denoised and corrected for amplicon errors using DADA2 v. 1.6 from QIIME 2 v. 2017.12.0 (Callahan et al., 2016). Default parameters were used for DADA2, except that the thread count parameter was set to 32 and the number of bases used for learning error patterns was set to 2,000,000. Reads with a phred quality score below 20 were removed. Two samples (CON cow d 7 of wk 5 and LAPF cow d 7 of wk 6) were removed for having a total read count less than 2 SD below the mean of all samples. Additionally, operational taxonomic units (**OTU**) that had a total read count of less than 3 across all samples were removed. Taxonomy was assigned using the 99% identity clustered file from Greengenes version 13.8 (DeSantis et al., 2006) at 99% identity. The output of DADA2, sample metadata, and phylogenic tree information were evaluated using Phyloseq 1.22.3 from Bioconductor 3.6 in R (https://cran.r-project.org/). The R script added an additional filtering step to remove OTU classified as rRNA with mitochondrial or chloroplast origin. Of the OTU, 98% could be classified to order, 65% to family, 30% to genus, and 2% to species level. All OTU from this data set aggregated into 72 families and 18 phyla for the rumen fluid samples, and fecal samples aggregated into 55 families and 13 phyla. Data files containing the number of normalized reads for each sample aggregated at the phylum and family levels were exported for further statistical analysis. Principal component analysis was conducted using DESeq2 v. 1.18.1 from Bioconductor 3.6 in R. Following library size normalization, the principal component analysis was calculated using the plotPCA procedure and visually rendered with ggplot2. Permutational multivariance ANOVA tests were conducted using adonis in ggplot2 to test the effect of treatment and sampling day on the overall microbiota.

Sample Size and Experimental Considerations

The primary outcome measures for experiment 1 were DMI, milk yield, and milk composition. Secondary outcome measures were nutrient digestion and fecal starch. Assuming an α of 0.05, a power of 0.80, and expected standard deviations, the study was adequately powered to detect a 2 kg/d change in milk yield and a 2.8 percentage unit change in DM digestion. For experiment 2, our primary outcome measures were rumen VFA and rumen and fecal pH and microbiota. Secondary outcome measures were DMI, milk yield, milk composition, and fecal starch. The number of animals was limited to 6 due to the budgetary constraints associated with rumen cannulation and sample collection and analysis. Based on typical standard errors we have observed in previous studies, we expected 6 animals to be sufficient to detect differences of 10 m*M*, 0.2, and 0.25 in rumen VFA, rumen pH, and fecal pH, respectively. Researchers were not blinded to treatments at any stage of the experimentation or sample analysis, because the quantitative outcome variables of interest were not expected to be influenced by human bias. No stopping rules were in place, and no interim analyses were conducted.

Statistical Analysis

Weekly means of DMI and milk yield were calculated for each cow in both experiments. Milk composition data for each day of sampling was calculated as the mean of the a.m. and p.m. sampling results weighted by milk yield at each milking.

Experiment 1. One cow (CON) was removed from the experiment during wk 8 due to clinical mastitis.

Before her removal from the experiment, she had an elevated SCC at every milk sampling, and her mean SCC over the course of the experiment was 1,591,000. Subclinical mastitis was also a problem for 4 other cows, 2 from CON (mean SCC 495,000 and 564,000) and 2 from LAPF (mean SCC 323,000 and 1,131,000). Data were analyzed both with and without the cows with chronic subclinical mastitis, and no differences in interpretation were detectable. Thus, all data were included in the statistical analyses, including data through wk 7 for the removed cow. Weekly measures of intake, milk yield, and milk composition were analyzed using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Somatic cell count (cells/mL) was converted to SCS ($\log_2$[SCC/100,000] + 3) before analysis to achieve homogeneity of residual variance. Treatment, week, and interaction of treatment by week were included as fixed effects. Data collected during the last week of the baseline period were included as covariates. The RANDOM _RESIDUAL_ statement was used to indicate repeated measures, the subject was cow nested within treatment, and an autoregressive covariance structure was used. Block and cow nested within block were included as random effects. Fecal score, fecal starch, apparent nutrient digestion, and body weight data were evaluated using the same model, except that fewer weeks were included in the model (covariate and wk 1, 2, 4, 6, 8, and 10 for fecal measures and total-tract nutrient digestion; covariate and wk 5 and 10 for body weight).

Experiment 2. All animals completed the study, and all results were included in the statistical analyses. Weekly measures of intake, milk yield, and milk composition were analyzed using the GLIMMIX procedure of SAS. Treatment, week, treatment sequence, period, parity, and the interactions of treatment by week and treatment by parity were included as fixed effects. The RANDOM _RESIDUAL_ statement was used to indicate repeated measures, the subject was the interaction of treatment and cow, and an autoregressive covariance structure was used. Cow within sequence was included as a random effect.

Daily mean intake, milk yield, and milk composition from d 7 of wk 5 and d 1, 3, and 7 of wk 6, and in situ TMR disappearance and fecal starch from d 7 of wk 5 and d 1 and 7 of wk 6, were separately analyzed to determine any short-term effects of high-starch feeding. These data were analyzed using the GLIMMIX procedure of SAS as described for the weekly means, except that the week term was replaced by an indicator of day, and data from d 7 of wk 5 were included as covariates.

Fecal pH and rumen VFA were analyzed using the GLIMMIX procedure of SAS. Treatment, day, hour, treatment sequence, period, parity, and the interactions of treatment by hour, treatment by day, treatment by day by hour, and treatment by parity were included as fixed effects. Data from d 7 of wk 5 were included as covariates. The RANDOM _RESIDUAL_ statement was used to indicate repeated measures, the subject was the interaction of treatment and cow, and an autoregressive covariance structure was used. Cow within sequence was included as a random effect.

Rumen pH data were used to calculate daily mean, minimum, and maximum pH, and minutes per day and area per day below pH 5.8. Those results were then analyzed separately for wk 5 (before the ration change) and 6 (following the ration change) using GLIMMIX in a model that included the fixed effects of treatment, day, parity, period, sequence, and the interaction of day by treatment.

Microbiota data collected on d 7 of wk 5 and d 1 and 7 of wk 6, aggregated to the phylum, family, and genus levels, were analyzed using GLIMMIX in a model that contained the fixed effects of treatment, day, treatment sequence, parity, and the interactions of treatment by day and treatment by parity. The RANDOM _RESIDUAL_ statement was used to indicate repeated measures, the subject was the interaction of treatment and cow, and an autoregressive covariance structure was used.

Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. All statistical analyses were pre-specified. For all models, when a significant effect of time (week or day) or a significant interaction of treatment by time occurred, the PDIFF option of the LSMEANS statement was used to determine differences among times or differences between treatments at individual times, respectively.

RESULTS AND DISCUSSION

Rations

The lower-starch ration fed in experiment 1 and wk 1 through 5 of experiment 2 was formulated to contain 16.7% CP, 30.2% NDF, 19.4% ADF, 25.0% starch, 7.6% ash, and 1.68 Mcal/kg of NE_L. The higher-starch ration fed in wk 6 of experiment 2 was formulated to contain 15.8% CP, 27.9% NDF, 17.7% ADF, 30.5% starch, 7.0% ash, and 1.72 Mcal/kg of NE_L. Analyzed CP, ash, and NE_L were similar to formulated values. Analyzed values of NDF and ADF were higher than formulated for both the normal-starch ration (by 2.1 and 2.4 percentage units, respectively) and the high-starch ration (by 0.7 and 1.3 percentage units, respectively; Table 1). Analyzed starch content was 1.2 percentage units

Table 2. Effects of treatment on performance measures in experiment 1 (n = 15 per treatment)

	Treatment[1]			*P*-value		
Item	CON	LAPF	SEM	Treatment	Week	Treatment × Week
DMI, kg/d	27.1	25.9	0.6	0.06	0.001	0.05
Milk, kg/d	45.9	45.8	1.0	0.94	0.001	0.95
Milk fat, %	3.83	3.60	0.11	0.14	0.008	0.32
Milk fat, kg/d	1.74	1.66	0.05	0.24	0.36	0.43
Milk protein, %	2.93	2.87	0.04	0.30	0.001	0.64
Milk protein, kg/d	1.33	1.30	0.02	0.23	0.001	0.41
ECM, kg/d	47.7	46.5	0.9	0.31	0.002	0.53
Milk/DMI, kg/kg	1.72	1.75	0.04	0.55	0.001	0.14
ECM/DMI, kg/kg	1.77	1.78	0.05	0.85	0.002	0.45
MUN, mg/dL	12.0	11.8	0.3	0.64	0.001	0.50
SCS	2.30	2.15	0.15	0.46	0.007	0.47
BW, kg	729	723	6	0.51	0.001	0.61

[1]Treatments were control (CON) or supplementation with a direct-fed microbial (LAPF, 28 g/d, providing 1 × 10^9 cfu/d of *Lactobacillus acidophilus* and 2 × 10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark).

lower than formulated for the lower-starch ration and 0.6 percentage units higher than formulated for the higher-starch ration.

Experiment 1

Treatment did not affect milk yield, milk composition, or body weight in experiment 1 (Table 2). We detected an interaction of treatment by week on DMI ($P = 0.05$). This was due to greater intakes by CON compared with LAPF cows during wk 4 (27.3 vs. 25.3 kg/d, $P = 0.01$) and wk 5 (28.4 vs. 25.9 kg/d, $P = 0.02$), and tendencies for greater intakes by CON than LAPF cows during wk 1 (26.4 vs. 25.0 kg/d, $P = 0.09$), 2 (26.8 vs. 25.2 kg/d, $P = 0.052$), and 3 (26.7 vs. 25.2 kg/d, $P = 0.09$), but no differences in wk 6 through 10 (data not shown). As a consequence, DMI overall tended to be lower for LAPF compared with CON ($P = 0.06$). It has been suggested that supplementing cows with LAB and LUB would provide a more consistent production of VFA, which can be used to support production (Nocek et al., 2003). Thus, we expected improved milk yield or productive efficiency in response to the LAPF treatment, but this was not observed. This was perhaps due to the relatively low animal numbers or the relatively high rates of subclinical mastitis. Previous work has demonstrated an increased milk yield when cows were fed the same supplement (Boyd et al., 2011; West and Bernard, 2011), although others observed no effect on milk yield (Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015). Similarly, some have observed an increase in productive efficiency (West and Bernard, 2011), but others have not (Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015). The 2 studies that reported no differences in performance (Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015) fed rations containing 24.1 or 24.5% starch, similar to the 23.8% starch ration fed in this study. It is possible that a higher-starch ration may challenge the rumen more and increase the likelihood of observing treatment differences. As reviewed by Krehbiel et al. (2003), responses to bacterial DFM supplementation are more consistent in beef cattle than dairy cattle, and this could be related to the greater rumen challenges presented by beef cattle compared with dairy cattle diets. The lack of effect of LAPF on milk composition in the current study is consistent with other work (Raeth-Knight et al., 2007; Boyd et al., 2011; West and Bernard, 2011; Ferraretto and Shaver, 2015).

Results for fecal score, fecal starch, and apparent total-tract nutrient digestion are presented in Table 3. Fecal score and digestion of DM, OM, CP, NDF, and ADF were not affected by treatment or the interaction of treatment by week. We detected an interaction of treatment by week for fecal starch ($P = 0.02$). This was due to greater fecal starch for CON than LAPF at wk 1 (0.76% vs. 0.44%, $P = 0.03$) and wk 2 (0.89% vs. 0.42%, $P = 0.002$) and a tendency for greater fecal starch for CON than LAPF at wk 4 (1.14% vs. 0.85%, $P = 0.06$) without differences at other times (data not shown). As a consequence of differences during those weeks, an overall effect of treatment on fecal starch occurred ($P = 0.03$). Apparent total-tract starch digestion tended to be higher ($P = 0.051$) for LAPF compared with CON, but the difference was very small.

We expected the LAPF treatment to improve digestibility of nutrients in the rumen that would be reflected by an increase in apparent total-tract nutrient digestion compared with CON. We also expected the improved digestibility to potentially manifest as an effect on fecal

Table 3. Effects of treatment on fecal measures and apparent total-tract nutrient digestibility in experiment 1 (n = 15 per treatment for fecal score and fecal starch; n = 7 per treatment for apparent digestibility)

	Treatment[1]			*P*-value		
Item	CON	LAPF	SEM	Treatment	Week[2]	Treatment × Week
Fecal score	2.95	3.08	0.07	0.19	0.001	0.41
Fecal starch, % DM	0.78	0.59	0.12	0.03	0.001	0.02
Apparent digestibility, %						
DM	68.8	69.2	0.3	0.39	0.001	0.46
OM	70.2	70.5	0.3	0.36	0.001	0.49
Starch	98.46	98.74	0.10	0.051	0.002	0.13
CP	69.2	69.3	0.4	0.92	0.001	0.27
NDF	42.0	42.7	0.7	0.53	0.001	0.45
ADF	39.7	40.5	0.7	0.46	0.001	0.25

[1]Treatments were control (CON) or supplementation with a direct-fed microbial (LAPF, 28 g/d, providing 1×10^9 cfu/d of *Lactobacillus acidophilus* and 2×10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark).

[2]Samples were collected at the end of wk 1, 2, 4, 6, 8, and 10.

score. Other than the minor effects on starch digestion and fecal starch, the lack of effect of treatment on digestion of other nutrients or fecal score suggests that nutrient digestibility between the 2 groups was similar. Results were mixed in other studies that evaluated supplementing a mixture of *Lactobacillus acidophilus* and *P. freudenreichii*, with Raeth-Knight et al. (2007) and Ferraretto and Shaver (2015) observing no effect and Boyd et al. (2011) observing increased CP, NDF, and ADF digestion in response to the DFM.

Experiment 2: Performance Data

No effect of treatment on intake, milk yield, or milk composition was detectable during the first 5 wk of each period in experiment 2 (Table 4), when cows were fed the same ration as in experiment 1. Although experiment 2 was underpowered to evaluate performance response during feeding of a normal-starch ration, these results support the findings of experiment 1. During wk 6, cows were abruptly switched to a higher-starch ration. Effects of this transition on performance are presented in Table 5, which evaluated data from d 1, 3, and 7 of wk 6, with data from d 7 of wk 5 included as covariates. We found no effect of treatment on DMI, milk protein, or SCS. Yields of milk and ECM were reduced for LAPF compared with CON ($P = 0.04$ and $P = 0.02$, respectively), and milk/DMI tended to be reduced for LAPF compared with CON ($P = 0.08$). Milk fat percentage tended to be affected by the interaction of treatment by day ($P = 0.06$) due to greater milk fat percentage for cows on the LAPF treatment on wk 6 d 1 compared with CON (4.08 vs. 3.62%). However, this interaction was primarily driven by greater milk fat percentages for LAPF cows from the morning milk sampling before exposure to the new diet (data not shown), and is thus likely not biologically relevant. Milk fat yield was affected by day ($P = 0.05$) and the interaction of treatment by day ($P = 0.06$), and tended to be affected by treatment ($P = 0.06$). The interaction was driven by greater fat yields for the LAPF treatment on d 1 of wk 6 (1.34 kg/d) and the CON treatment on d 3 of wk 6 (1.35 kg/d) than for the LAPF treatment on d 3 of wk 6 (1.15 kg/d) or d 7 of wk 6 (1.12 kg/d; $P < 0.05$; data not shown). As a consequence of the treatment by day interaction on fat yield, we detected a tendency for an interaction of treatment by day on ECM yield ($P = 0.07$). Day affected MUN ($P = 0.02$), which was lower on d 3 of wk 6 (8.5 mg/dL) compared with the other days (10.1 to 10.2 mg/dL; $P = 0.01$; data not shown).

We had hypothesized that LAPF would stabilize the rumen environment following the abrupt shift to the higher-starch ration and manifest as improved milk yield, milk composition, or feed efficiency during the transition compared with CON. Counter to our hypothesis, this was not observed, and the treatment effects that were observed indicated improved performance for CON compared with LAPF. This may have been a result of failure of the ration shift to adequately challenge the rumen with the high-starch ration, as will be described.

Experiment 2: Rumen pH and VFA

Rumen pH was measured continuously during wk 5 and 6, and results for each week are presented in Table 6. When cows were fed the 23.8% starch ration during wk 5, we detected no effects of treatment or the interaction of treatment by week on any rumen pH variables. When cows were abruptly switched to the 31.1% starch ration during wk 6, we expected to observe a decrease

Table 4. Effects of treatment on performance measures during the 5 wk of feeding the 23.8% starch diet in experiment 2 (n = 6)

	Treatment[1]			*P*-value		
Item	CON	LAPF	SEM	Treatment	Week	Treatment × Week
DMI, kg/d	23.5	23.5	3.1	0.92	0.17	0.94
Milk, kg/d	34.1	35.0	6.9	0.38	0.47	0.96
Milk fat, %	3.57	3.63	0.28	0.52	0.78	0.77
Milk fat, kg/d	1.18	1.22	0.24	0.26	0.59	0.63
Milk protein, %	2.98	2.98	0.17	0.90	0.34	0.23
Milk protein, kg/d	0.99	1.01	0.18	0.48	0.94	0.37
ECM, kg/d	33.9	34.9	6.7	0.27	0.56	0.59
Milk/DMI, kg/kg	1.43	1.45	0.19	0.56	0.02	0.70
ECM/DMI, kg/kg	1.43	1.45	0.18	0.50	0.21	0.51
MUN, mg/dL	10.9	10.8	0.4	0.80	0.006	0.98
SCS	2.05	1.98	0.57	0.75	0.07	0.27

[1]Treatments were control (CON) or supplementation with a direct-fed microbial (LAPF, 28 g/d, providing 1 × 10^9 cfu/d of *Lactobacillus acidophilus* and 2 × 10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark).

in rumen pH. However, this did not occur, as all pH measures were numerically greater during wk 6 than wk 5 (Table 6). The lack of a rumen pH decrease in response to the shift suggests that we failed to adequately challenge the rumen. This may have been the result of using ground corn grain as the vehicle to increase dietary starch instead of a more rapidly fermentable starch source. Despite this lack of response to feeding the higher-starch ration, the LAPF treatment increased both mean pH ($P = 0.006$) and minimum pH ($P = 0.02$) during wk 6. We had expected the LAPF treatment to stabilize rumen fermentation during the ration change. Although LAPF increased rumen pH in wk 6, we cannot conclude that the treatment stabilized the rumen, due to lack of evidence that the higher-starch ration challenged the rumen. Similar to our findings, a study in dairy cattle fed a 24% starch diet found no effect of supplementation with *L. acidophilus* and *P. freudenreichii* on rumen pH (Raeth-Knight et al., 2007), and work in beef cattle fed an 87% barley ration found no effect of *P. freudenreichii* alone or combined with *Enterococcus faecium* on rumen pH (Ghorbani et al., 2002). However, others have reported that LAB can modify rumen pH in cattle fed high-starch diets.

Table 5. Effects of treatment on performance measures, 24 h in situ TMR digestibility, and fecal starch during the transition from the 23.8% starch diet during wk 5 to the 31.1% starch diet during wk 6 in experiment 2 (n = 6)

	Treatment[1]			*P*-value		
Item	CON	LAPF	SEM	Treatment	Day[2]	Treatment × Day
DMI, kg/d	24.8	24.4	0.5	0.55	0.83	0.13
Milk, kg/d	35.7	33.2	0.7	0.04	0.30	0.65
Milk fat, %	3.68	3.68	0.12	0.97	0.10	0.06
Milk fat, kg/d	1.27	1.20	0.03	0.06	0.05	0.04
Milk protein, %	3.02	2.98	0.19	0.35	0.97	0.36
Milk protein, kg/d	1.06	0.96	0.04	0.11	0.50	0.49
ECM, kg/d	36.2	33.7	0.6	0.02	0.12	0.07
Milk/DMI, kg/kg	1.42	1.32	0.03	0.08	0.64	0.11
ECM/DMI, kg/kg	1.44	1.35	0.04	0.11	0.26	0.85
MUN, mg/dL	9.7	9.5	0.2	0.43	0.02	0.27
SCS	1.70	1.97	0.32	0.55	0.11	0.55
24-h in situ DM disappearance, %	76.2	75.8	1.7	0.83	0.03	0.68
Fecal starch, % of DM	2.15	2.97	0.21	0.06	0.19	0.96

[1]Treatments were control (CON) or supplementation with a direct-fed microbial (LAPF, 28 g/d, providing 1 × 10^9 cfu/d of *Lactobacillus acidophilus* and 2 × 10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark).

[2]For intake and milk measures, data were from d 1, 3, and 7 of wk 6. Rumen in situ disappearance of TMR and fecal starch were determined on d 1 and 7 of wk 6. For all models, data from d 7 of wk 5 were included as covariates.

Table 6. Effects of treatment on rumen pH in experiment 2 (n = 6)

	Treatment[1]			*P*-value		
Item	CON	LAPF	SEM	Treatment	Day[2]	Treatment × Day
Week 5						
Mean pH	6.28	6.27	0.07	0.84	0.03	0.90
Minimum pH	5.81	5.80	0.07	0.85	0.70	0.50
Maximum pH	6.80	6.80	0.04	0.94	0.002	0.16
Min/d below pH 5.8	78	69	53	0.90	0.64	0.82
Area/d below pH 5.8	10.9	12.3	7.3	0.88	0.78	0.89
Week 6						
Mean pH	6.33	6.42	0.09	0.006	0.62	0.82
Minimum pH	5.87	5.97	0.08	0.02	0.22	0.97
Maximum pH	6.86	6.90	0.07	0.36	0.10	0.40
Min/d below pH 5.8	30	11	24	0.15	0.65	0.80
Area/d below pH 5.8	3.3	1.0	2.6	0.25	0.57	0.78

[1]Treatments were control (CON) or supplementation with a direct-fed microbial (LAPF, 28 g/d, providing 1 × 10^9 cfu/d of *Lactobacillus acidophilus* and 2 × 10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark).

[2]Rumen pH was measured continuously during wk 5 to 6. Cows were fed a 23.8% starch diet during wk 5 and a 31.1% starch diet during wk 6. Day was d 1 to 7 of each week.

Supplementation with LAB alone has some ability to increase rumen pH during SARA induction in dairy cows (Chiquette et al., 2015), and LAB tended to increase rumen pH over time in steers fed a finishing diet (Kenney et al., 2015). On the other hand, a high dose of *Propionibacterium* strain P169 decreased rumen pH in lactating cows (Stein et al., 2006).

The only rumen VFA affected by treatment was isovalerate (Table 7). Rumen isovalerate tended to be affected by both treatment ($P = 0.06$) and the interaction of treatment by day ($P = 0.06$). This was due to greater isovalerate on d 7 of wk 6 for CON than for LAPF (2.10 vs. 1.60 m*M*; $P = 0.01$; data not shown), with isovalerate on d 1 of wk 6 being intermediate (1.81 and 1.78 m*M* for CON and LAPF, respectively). Day affected butyrate ($P = 0.02$) and valerate ($P = 0.04$) and tended to affect acetate ($P = 0.09$). In all cases this was due to greater concentration during d 7 of wk 6 than wk d 1 of 6 (13.5 vs. 11.5 m*M* for butyrate, 1.73 vs. 1.51 mM for valerate, and 73.6 vs. 66.4 m*M* for acetate). The increase in those rumen VFA from the first day of the diet switch to the final day after the

Table 7. Effects of treatment on rumen organic acids and fecal pH in experiment 2 (n = 6)

	Treatment[1]			*P*-value[2]		
Item	CON	LAPF	SEM	Treatment	Day[3]	Hour[3]
Rumen organic acid, m*M*						
Acetate	71.9	68.0	3.1	0.39	0.09	0.001
Propionate	25.5	24.8	2.9	0.80	0.12	0.001
Butyrate	12.8	12.3	0.7	0.57	0.02	0.001
Isobutyrate	1.29	1.25	0.04	0.47	0.14	0.95
Valerate	1.61	1.64	0.11	0.82	0.04	0.001
Isovalerate	1.96	1.69	0.13	0.06	0.64	0.002
Lactate[4]	0.37	0.32	0.08	0.55	0.36	0.04
Total organic acids[5]	117.3	109.4	6.2	0.37	0.12	0.001
Fecal pH	6.80	6.82	0.04	0.63	0.001	0.27

[1]Treatments were control (CON) or supplementation with a direct-fed microbial (LAPF, 28 g/d, providing 1 × 10^9 cfu/d of *Lactobacillus acidophilus* and 2 × 10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark).

[2]Interactions of treatment × hour, treatment × day, day × hour, or treatment × day × hour were not observed for any variables ($P \geq 0.10$) except for isovalerate ($P = 0.06$ for treatment × day).

[3]Rumen and fecal samples were collected 4, 10, 16, and 22 h after feeding on d 7 of wk 5 and d 1 and 7 of wk 6. Data from d 1 and 7 of wk 6 were evaluated in a model that included data from d 7 of wk 5 as covariates.

[4]Lactate was log-transformed before statistical analyses. LSM and SEM presented in the table were back-transformed.

[5]Total organic acids = total VFA + lactate.

transition indicates that the higher-starch feeding during wk 6 did affect rumen VFA, despite not affecting rumen pH.

In this experiment, we hypothesized that the LAPF treatment would increase ruminal propionate directly due to the addition of *P. freudenreichii* and indirectly via lactic acid produced by *L. acidophilus* serving as a substrate for propionate-producing bacteria. However, the lack of a treatment effect on ruminal lactate or propionate suggests that supplemented bacterial strains did not alter rumen VFA production. Some studies that fed *Propionibacterium* species alone have reported increases in rumen propionate (Stein et al., 2006; Lehloenya et al., 2008; Weiss et al., 2008). However, other studies that fed LAB alone or a combination of *L. acidophilus* and *P. freudenreichii* found no effect on rumen propionate in vitro (Meissner et al., 2014) or in vivo (Raeth-Knight et al., 2007; Kenney et al., 2015).

Experiment 2: In Situ Digestion, Fecal pH, and Fecal Starch

In situ TMR DM disappearance following 24 h of rumen incubation was not affected by treatment or the interaction of treatment by day (Table 5). Dry matter disappearance was also measured following 6, 12, and 18 h of rumen incubation, and those were also not affected by treatment or the interaction of treatment by day ($P > 0.10$; data not shown). We detected an effect of day on 24 h in situ DM disappearance due to greater disappearance on d 7 of wk 6 (78.1%) than on d 1 of wk 6 (73.8%; $P = 0.03$). We had expected the LAPF treatment to increase in situ DM disappearance, but this was not observed. However, this supports the findings of minimal changes in total-tract apparent digestion observed in experiment 1. We are unaware of other work evaluating in situ response to *L. acidophilus* and *P. freudenreichii*, but in situ digestion of feed ingredients was not affected by *Propionibacterium* alone or *Propionibacterium* plus *E. faecium* in beef steers (Ghorbani et al., 2002). On the contrary, Nocek and Kautz (2006) reported increased in situ digestion of forages in cows supplemented with yeast and *Enterococcus* strains.

Fecal samples were collected at 4, 10, 16, and 22 h after feeding on d 7 of wk 5 and d 1 and 7 of wk 6. Fecal pH was measured at all of those time points, and remaining fecal samples were composited by day for each cow for measurement of fecal starch. The model evaluated wk 6 effects using wk 5 d 7 results as covariates. We detected no effect of treatment on fecal pH ($P = 0.63$; Table 7). An effect of day was observed ($P = 0.001$), and fecal pH was lower on d 7 (6.73) than d 1 of wk 6 (6.90), suggesting that the higher-starch diet increased postruminal starch flow and consequent intestinal fermentation. Fecal starch tended to be affected by treatment ($P = 0.06$; Table 5). Counter to our hypothesis and to the results of experiment 1, fecal starch was greater for LAPF (2.97%) than for CON (2.15%), suggesting that LAPF reduced starch digestion compared with CON. However, even following the challenge, fecal starch remained below 3%, again indicating a failure of the model to adequately alter starch digestion.

Experiment 2: Rumen and Fecal Bacterial Composition

The principal component analysis plots of the bacterial composition of the rumen fluid and the fecal samples are presented in Figures 1A and 1B, respectively. No clusters based on treatment or dietary starch content were apparent. This was confirmed by the adonis test, which indicated no effect of treatment or sampling day on principal component analysis of rumen fluid ($P = 0.51$ and $P = 0.36$, respectively) or feces ($P = 0.84$ and $P = 0.50$, respectively).

Bacterial phyla with at least 1% abundance in rumen fluid and fecal samples are presented in Supplemental Tables S1 and S2 (http://dx.doi.org/10.17632/tv5rc7yccr.1). The interaction of treatment by day did not affect any of the phyla presented ($P > 0.10$). Only the least-abundant phylum presented, *Actinobacteria*, was affected by treatment, with lower relative abundance in CON compared with LAPF ($P = 0.01$). Day affected relative abundance of *Bacteroidetes* and *Firmicutes* ($P = 0.03$ and $P = 0.04$, respectively) and tended to affect *Tenericutes* ($P = 0.052$) and *Actinobacteria* ($P = 0.09$). For *Bacteroidetes*, this was due to higher abundance during feeding of the 23.8% starch ration on d 7 of wk 5 (73.5%) than on the first (wk 6 d 1) or seventh (wk 6 d 7) day after the transition to the 31.1% starch ration (65.0% on both days). The opposite was observed for *Firmicutes*, with lower abundance on d 7 of wk 5 (21.3%) than on d 1 or d 7 of wk 6 (28.8% on both days). As reviewed by Khafipour et al. (2016), increased *Firmicutes* and decreased *Bacteroidetes* are commonly observed when feeding higher-starch diets, and the results from the current study suggest that this shift occurred within 24 h of feeding the higher-starch ration. For *Tenericutes* and *Actinobacteria*, the trends were due to lower abundance on d 7 of wk 5 (1.70% and 0.85%, respectively) than on d 1 of wk 6 for *Tenericutes* (3.08%, $P = 0.02$) or d 7 of wk 6 for *Actinobacteria* (1.64%, $P = 0.03$), with the other day in wk 6 being intermediate (2.87% for *Tenericutes* and 1.08% for *Actinobacteria*).

Only 4 bacterial phyla had at least 1% relative abundance in fecal samples (Supplemental Table S2).

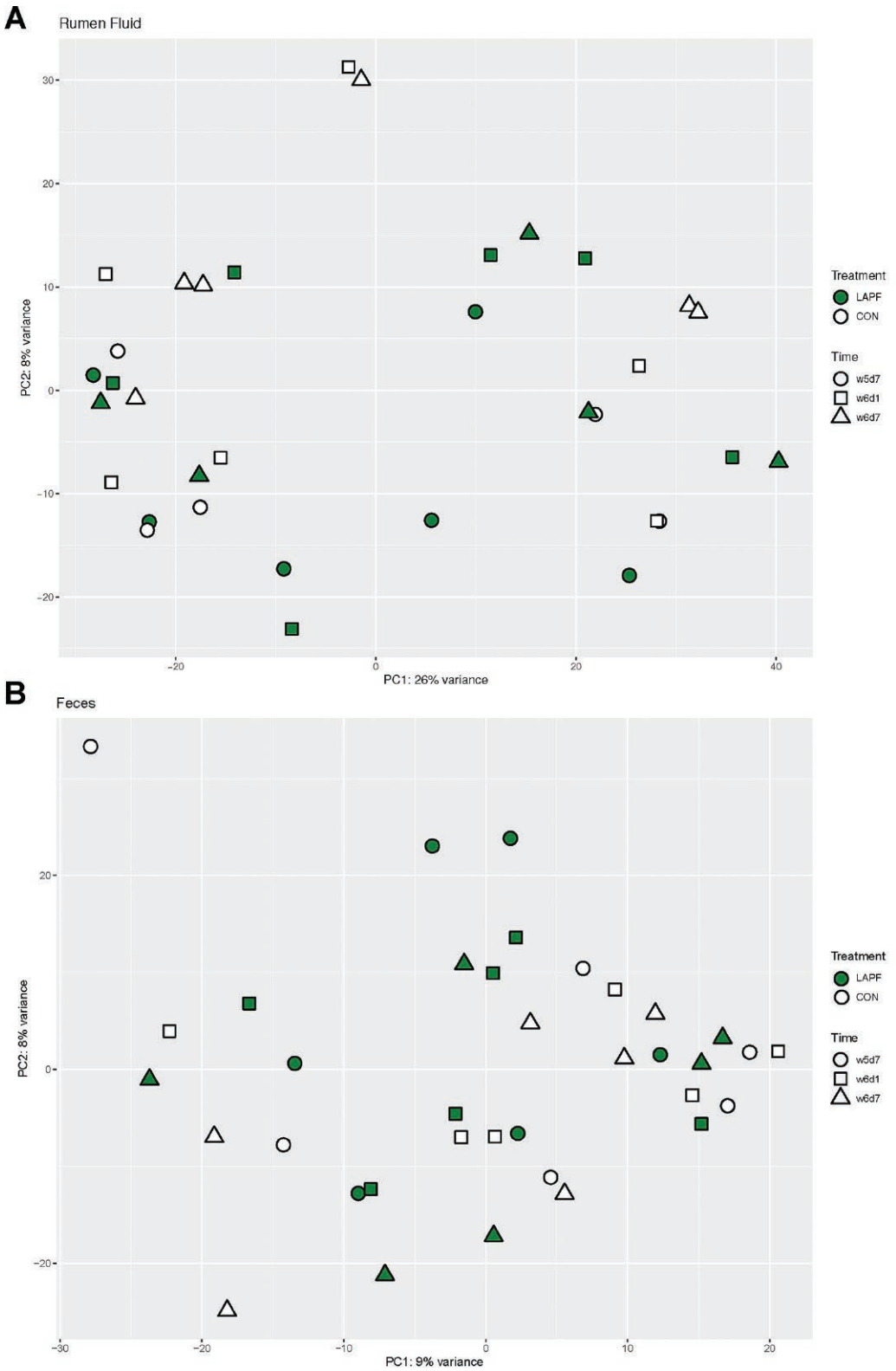

Figure 1. Principal component (PC) analysis plots of bacteria detected in rumen fluid (A) and fecal samples (B). Treatments were control (CON, white) or supplementation with a direct-fed microbial (LAPF, green, 28 g/d, providing 1×10^9 cfu/d of *Lactobacillus acidophilus* and 2×10^9 cfu/d of *Propionibacterium freudenreichii*; Bovamine, Chr. Hansen, Hørsholm, Denmark). Samples for microbiota analysis were collected on d 7 of wk 5 following feeding of the 23.8% starch ration (circles, w5d7) and on d 1 (squares, w6d1) and 7 (triangles, w6d7) of wk 6 following feeding of the 31.1% starch ration.

Those phyla with the greatest abundance, *Firmicutes* and *Bacteroidetes*, were not affected by treatment, day, or their interaction, and represented approximately 51% and 42% of fecal bacteria phyla, respectively. This is in contrast to Plaizier et al. (2017), who observed increased *Firmicutes* in feces 7 or 10 d following feeding of a high-starch ration to induce subacute ruminal acidosis. However, similar to these results, both Plaizier et al. (2017) and Mao et al. (2012), who sampled feces 12 to 21 d following feeding of a SARA-inducing ration, reported no effect of a high-starch ration on fecal *Bacteroidetes*. *Tenericutes* tended to be affected by the interaction of treatment by day ($P = 0.09$), and *Spirochaetes* was affected by day ($P = 0.03$). For *Tenericutes*, the tendency for the interaction was due to numerically greater abundance on d 7 of wk 5 for the LAPF treatment (4.05%) than for d 1 of wk 6 for the LAPF treatment and d 7 of wk 5 and d 7 of wk 6 of the CON treatment (2.39–2.58%; $P \geq 0.09$). The day effect for *Spirochaetes* was due to greater relative abundance on d 7 of wk 6 (2.30%) than on d 7 of wk 5 or d 1 of wk 6 (1.26% and 1.19%, respectively; $P \leq 0.04$). Because there would be little time for the dietary shift to affect fecal microbial populations between d 7 of wk 5 and d 1 of wk 6, the similarity in *Spirochaetes* relative abundance on those days compared with increased relative abundance at d 7 of wk 6 suggests that this occurred in response to the higher-starch ration. Because *Spirochaetes* are carbohydrate-fermenting bacteria (Canale-Parola, 1977), this suggests that the higher-starch diet may have increased carbohydrate passage to and fermentation in the intestines. However, the relative abundance of fecal *Spirochaetes* was not affected by a subacute ruminal acidosis challenge in other cow studies (Mao et al., 2012; Plaizier et al., 2017).

Those families with at least 1% abundance in rumen fluid and fecal samples are presented in Supplemental Tables S3 and S4 (http://dx.doi.org/10.17632/tv5rc7yccr.1). For rumen fluid, *Prevotellaceae* was the most dominant bacterial family, representing over 50% of the reads. Others have similarly found *Prevotellaceae* to be the most dominant family (Paz et al., 2016; De Mulder et al., 2017; Castillo-Lopez et al., 2018). *Prevotellaceae* was affected by day ($P = 0.003$) and tended to be affected by the interaction of treatment by day ($P = 0.06$). The interaction was primarily driven by LAPF having greater abundance of *Prevotellaceae* than CON on d 7 of wk 5 during the moderate-starch feeding (63.7% vs. 56.7%) and numerically lower abundance on d 1 (44.9% vs. 50.8%) and d 7 of wk 6 (47.6% vs. 53.5%). The day effect was due to lower *Prevotellaceae* on d 1 of wk 6 (47.8%) and d 7 of wk 6 (50.3%) than on d 7 of wk 5 (60.2%; $P \leq 0.01$). This is in contrast with McCann et al. (2016), who observed increased *Prevotellaceae* following a subacute ruminal acidosis challenge. Family *Lachnospiraceae* tended to be affected by day ($P = 0.09$) due to greater abundance on d 7 of wk 6 (4.07%) than on d 7 of wk 5 (2.80%, $P = 0.03$), with d 1 of wk 6 being intermediate (3.45%). *Lachnospiraceae* metabolize carbohydrates, including starch (Vacca et al., 2020), which could explain the tendency for the increased abundance upon transition to the higher-starch ration. Day also affected order *Bacteroidales*, uncultured family *S24–7* ($P = 0.001$), due to lower concentrations on d 7 of wk 5 (2.25%) than wk 6 d 1 (4.07%) or wk 6 d 7 (3.93%; $P \leq 0.003$). McCann et al. (2016) similarly observed an increase in *S24–7* following higher-starch feeding during a subacute ruminal acidosis challenge. For the unclassified family within class *Mollicutes* and order *RF39*, the day effect ($P = 0.048$) was due to lower concentrations on d 7 of wk 5 (1.70%) than on d 1 of wk 6 (3.13%, $P = 0.02$), with d 7 of wk 6 being intermediate (2.94%). No other effects of treatment, day, or their interaction were observed on rumen microbial families with over 1% abundance.

For feces, the most abundant family was *Ruminococcaceae*, as has been reported by others (Rice et al., 2012; Tang et al., 2017), but it was not affected by treatment, day, or interaction of treatment and day (Supplemental Table S4; http://dx.doi.org/10.17632/tv5rc7yccr.1). Family *Bacteroidaceae* tended to be affected by the interaction of treatment and day ($P = 0.08$). This was due to relative abundance of *Bacteroidaceae* in cows on the LAPF treatment on d 1 of wk 6 (12.1%) being numerically greater than cows on the LAPF treatment on other days (8.5–8.9%) as well as cows on the CON treatment on any day (7.0–9.1%). Cows on the LAPF treatment had greater relative abundance of fecal *Rikenellaceae* than cows on the CON treatment ($P = 0.03$). In addition, we detected a tendency for an effect of day ($P = 0.09$) due to lower relative abundance on d 7 of wk 5 (6.34%) than on d 7 of wk 6 (8.10%; $P = 0.03$), with d 1 of wk 6 being intermediate (7.37%). Similarly, the effect of day observed for both order *Bacteroidales* family RF16 and family *Spirochaetaceae* was due to lower relative abundance on d 7 of wk 5 (1.43% and 1.08%, respectively) than on d 7 of wk 6 (2.57% and 2.01%, respectively; $P = 0.01$), and d 1 of wk 6 was intermediate (1.67% and 1.26%, respectively). We detected an interaction of treatment by day for family *Clostridiaceae* ($P = 0.003$). Relative abundance of *Clostridiaceae* did not differ by day for cows on the LAPF treatment (1.11–1.36%), but, for cows on the CON treatment, relative abundance was lower on d 7 of wk 5 (0.83%) and d 7 of wk 6 (1.04%) than on d 1 of wk 6 (1.65%; $P < 0.01$).

Genus-level results for rumen fluid and feces are presented in Supplemental Tables S5 and S6 (http:/

/dx.doi.org/10.17632/tv5rc7yccr.1), respectively. Only 30% of OTU could be classified to genus, which limits interpretations, but the findings overall were similar to those at the family level. The main additional finding is that *Bifidobacterium* was increased by the LAPF treatment in rumen fluid ($P = 0.02$).

CONCLUSIONS

In experiment 1, supplementation of dairy cow diets with a mixture of *Lactobacillus animalis* and *Propionibacterium freudenreichii* did not affect animal performance. A tendency for a slight increase in apparent total-tract starch digestion was detectable compared with control, but this was very small, and digestion of other nutrients was unaffected. In experiment 2, the bacterial direct-fed microbial increased fecal starch concentration. A few modest changes in rumen and fecal bacterial populations were observed.

ACKNOWLEDGMENTS

The authors thank Chr. Hansen Animal Health and Nutrition (Hørsholm, Denmark) for financial support of this work. The authors have not stated any other conflicts of interest.

REFERENCES

AOAC International. 2000. Official Methods of Analysis of AOAC International. 17th ed. Association of Official Analytical Chemists, Washington, DC.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brejnrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodríguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gauglitz, S. M. Gibbons, D. L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G. A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B. D. Kaehler, K. B. Kang, C. R. Keefe, P. Keim, S. T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M. G. I. Langille, J. Lee, R. Ley, Y.-X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B. D. Martin, D. McDonald, L. J. McIver, A. V. Melnik, J. L. Metcalf, S. C. Morgan, J. T. Morton, A. T. Naimey, J. A. Navas-Molina, L. F. Nothias, S. B. Orchanian, T. Pearson, S. L. Peoples, D. Petras, M. L. Preuss, E. Pruesse, L. B. Rasmussen, A. Rivers, M. S. Robeson II, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S. J. Song, J. R. Spear, A. D. Swafford, L. R. Thompson, P. J. Torres, P. Trinh, A. Tripathi, P. J. Turnbaugh, S. Ul-Hasan, J. J. J. van der Hooft, F. Vargas, Y. Vázquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K. C. Weber, C. H. D. Williamson, A. D. Willis, Z. Z. Xu, J. R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, and J. G. Caporaso. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852–857. https://doi.org/10.1038/s41587-019-0209-9.

Boyd, J., J. W. West, and J. K. Bernard. 2011. Effects of the addition of direct-fed microbials and glycerol to the diet of lactating dairy cows on milk yield and apparent efficiency of yield. J. Dairy Sci. 94:4616–4622. https://doi.org/10.3168/jds.2010-3984.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. Johnson, and S. P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13:581–583. https://doi.org/10.1038/nmeth.3869.

Canale-Parola, E. 1977. Physiology and evolution of spirochetes. Bacteriol. Rev. 41:181–204. https://doi.org/10.1128/MMBR.41.1.181-204.1977.

Castillo-Lopez, E., J. Moats, N. D. Aluthge, H. A. Ramirez Ramirez, D. A. Christensen, T. Mutsvangwa, G. B. Penner, and S. C. Fernando. 2018. Effect of partially replacing a barley-based concentrate with flaxseed-based products on the rumen bacterial population of lactating Holstein dairy cows. J. Appl. Microbiol. 124:42–57. https://doi.org/10.1111/jam.13630.

Chiquette, J., J. Lagrost, C. L. Girard, G. Talbot, S. Li, J. C. Plaizier, and I. K. Hindrichsen. 2015. Efficacy of the direct-fed microbial *Enterococcus faecium* alone or in combination with *Saccharomyces cerevisiae* or *Lactococcus lactis* during induced subacute ruminal acidosis. J. Dairy Sci. 98:190–203. https://doi.org/10.3168/jds.2014-8219.

De Mulder, T., K. Goossens, N. Peiren, L. Vandaele, A. Haegeman, C. De Tender, T. Ruttink, T. V. de Wiele, and S. De Campeneere. 2017. Exploring the methanogen and bacterial communities of rumen environments: Solid adherent, fluid and epimural. FEMS Microbiol. Ecol. 93. https://doi.org/10.1093/femsec/fiw251.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72:5069–5072. https://doi.org/10.1128/AEM.03006-05.

Ferraretto, L. F., and R. D. Shaver. 2015. Effect of direct-fed microbial supplementation on lactation performance and total-tract starch digestibility by midlactation dairy cows. Prof. Anim. Sci. 31:63–67. https://doi.org/10.15232/pas.2014-01369.

Ghorbani, G. R., D. P. Morgavi, K. A. Beauchemin, and J. A. Z. Leedle. 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. J. Anim. Sci. 80:1977–1985. https://doi.org/10.2527/2002.8071977x.

Goering, H. K., and P. J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). In Agricultural Handbook no. 379. USDA ARS, Washington, DC.

Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49. https://doi.org/10.1093/jaoac/92.1.42.

Hulsen, J. 2006. Cow Signals: The Practical Guide for Dairy Cow Management. Vetvice, Bergen op Zoom, the Netherlands.

Kenney, N. M., E. S. Vanzant, D. L. Harmon, and K. R. McLeod. 2015. Direct-fed microbials containing lactate-producing bacteria influence ruminal fermentation but not lactate utilization in steers fed a high-concentrate diet. J. Anim. Sci. 93:2336–2348. https://doi.org/10.2527/jas.2014-8570.

Khafipour, E., S. Li, H. M. Tun, H. Derakhshani, S. Moossavi, and J. C. Plaizier. 2016. Effects of grain feeding on microbiota in the digestive tract of cattle. Anim. Front. 6:13–19. https://doi.org/10.2527/af.2016-0018.

Krehbiel, C. R., S. R. Rust, G. Zhange, and S. E. Gilliland. 2003. Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. J. Anim. Sci. 81(E Suppl. 2):E120–E132. https://doi.org/10.2527/2003.8114_suppl_2E120x.

Lehloenya, K. V., C. R. Krehbiel, K. J. Mertz, T. G. Rehberger, and L. J. Spicer. 2008. Effects of propionibacteria and yeast culture fed to steers on nutrient intake and site and extent of digestion. J. Dairy Sci. 91:653–662. https://doi.org/10.3168/jds.2007-0474.

Mao, S., R. Zhang, D. Wang, and W. Zhu. 2012. The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. BMC Vet. Res. 8:237. https://doi.org/10.1186/1746-6148-8-237.

McAllister, T. A., K. A. Beauchemin, A. Y. Alazzeh, J. Baah, R. M. Teather, and K. Stanford. 2011. Review: The use of direct fed mi-

crobials to mitigate pathogens and enhance production in cattle. Can. J. Anim. Sci. 91:193–211. https://doi.org/10.4141/cjas10047.
McCann, J. C., S. Luan, F. C. Cardoso, H. Derakhshani, E. Khafipour, and J. J. Loor. 2016. Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.00701.
Meissner, H. H., P. H. Henning, K. J. Leeuw, F. M. Hagg, C. H. Horn, A. Kettunen, and J. H. A. Apajalahti. 2014. Efficacy and mode of action of selected non-ionophore antibiotics and direct-fed microbials in relation to *Megasphaera elsdenii* NCIMB 41125 during in vitro fermentation of an acidosis-causing substrate. Livest. Sci. 162:115–125. https://doi.org/10.1016/j.livsci.2014.01.026.
Muck, R. E., and J. T. Dickerson. 1988. Storage-temperature effects on proteolysis in alfalfa silage. Trans. ASAE 31:1005–1009. https://doi.org/10.13031/2013.30813.
Nocek, J. E., and W. P. Kautz. 2006. Direct-fed microbial supplementation on ruminal digestion, health, and performance of pre- and postpartum dairy cattle. J. Dairy Sci. 89:260–266. https://doi.org/10.3168/jds.S0022-0302(06)72090-2.
Nocek, J. E., W. P. Kautz, J. A. Z. Leedle, and E. Block. 2003. Direct-fed microbial supplementation on the performance of dairy cattle during the transition period. J. Dairy Sci. 86:331–335. https://doi.org/10.3168/jds.S0022-0302(03)73610-8
NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
Paz, H. A., C. L. Anderson, M. J. Muller, P. J. Kononoff, and S. C. Fernando. 2016. Rumen bacterial community composition in Holstein and Jersey cows is different under same dietary condition and is not affected by sampling method. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01206.
Plaizier, J. C., S. C. Li, A. M. Danscher, H. Derakshani, P. H. Andersen, and E. Khafipour. 2017. Changes in microbiota in rumen digesta and feces due to a grain-based subacute ruminal acidosis (SARA) challenge. Microb. Ecol. 74:485–495. https://doi.org/10.1007/s00248-017-0940-z.
Raeth-Knight, M. L., J. G. Linn, and H. G. Jung. 2007. Effect of direct-fed microbials on performance, diet digestibility, and rumen characteristics of Holstein dairy cows. J. Dairy Sci. 90:1802–1809. https://doi.org/10.3168/jds.2006-643.
Rice, W. C., M. L. Galyean, S. B. Cox, S. E. Dowd, and N. A. Cole. 2012. Influence of wet distillers grains diets on beef cattle fecal bacterial community structure. BMC Microbiol. 12:25. https://doi.org/10.1186/1471-2180-12-25.
Seo, J. K., S. W. Kim, M. H. Kim, S. D. Upadhaya, D. K. Kam, and J. K. Ha. 2010. Direct-fed microbials for ruminant animals. Asian-Australas. J. Anim. Sci. 23:1657–1667. https://doi.org/10.5713/ajas.2010.r.08.
Stein, D. R., D. T. Allen, E. B. Perry, J. C. Bruner, K. W. Gates, T. G. Rehberger, K. Mertz, D. Jones, and L. J. Spicer. 2006. Effects of feeding propionibacteria to dairy cows on milk yield, milk components, and reproduction. J. Dairy Sci. 89:111–125. https://doi.org/10.3168/jds.S0022-0302(06)72074-4.
Tang, M. T., H. Y. Han, Z. Yu, T. Tsuruta, and N. Nishino. 2017. Variability, stability, and resilience of fecal microbiota in dairy cows fed whole crop corn silage. Appl. Microbiol. Biotechnol. 101:6355–6364. https://doi.org/10.1007/s00253-017-8348-8.
Vacca, M., G. Celano, F. M. Calabrese, P. Portincasa, M. Gobbetti, and M. De Angelis. 2020. The controversial role of human gut *Lachnospiraceae*. Microorganisms 8:573. https://doi.org/10.3390/microorganisms8040573.
Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.
Weiss, W. P., D. J. Wyatt, and T. R. McKelvey. 2008. Effect of feeding propionibacteria on milk production by early lactation dairy cows. J. Dairy Sci. 91:646–652. https://doi.org/10.3168/jds.2007-0693.
West, J. W., and J. K. Bernard. 2011. Effects of addition of bacterial inoculants to the diets of lactating dairy cows on feed intake, milk yield, and milk composition. Prof. Anim. Sci. 27:122–126. https://doi.org/10.15232/S1080-7446(15)30458-7.
Wisener, L. V., J. M. Sargeant, A. M. O'Connor, M. C. Faires, and S. K. Glass-Kaastra. 2015. The use of direct-fed microbials to reduce shedding of *Escherichia coli* O157 in beef cattle: A systematic review and meta-analysis. Zoonoses Public Health 62:75–89. https://doi.org/10.1111/zph.12112.
Yoon, I. K., and M. D. Stern. 1995. Influence of direct-fed microbials on ruminal microbial fermentation and performance of ruminants: A review. Asian Aust. J. Anim. Sci. 8:533–555. https://doi.org/10.5713/ajas.1995.553.

ORCIDS

S. Polukis https://orcid.org/0000-0002-6419-5826
M. A. Miller https://orcid.org/0000-0001-9076-6066
L. Kung Jr. https://orcid.org/0000-0001-6481-7966
T. F. Gressley https://orcid.org/0000-0002-6509-3004