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Gastrointestinal Biology

Cecal microbiota of feedlot cattle fed a four-species *Bacillus* supplement

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Abstract

As commercial fed cattle consume large amounts of concentrate feedstuffs, hindgut health can be challenged. The objective of this study was to evaluate the effects of a commercially available *Bacillus* feed additive on cattle health outcomes and cecal microbiota of fed cattle at the time of harvest. Commercial cattle from a single feedlot were identified for characterization of cecal microbial communities using 16S ribosomal ribonucleic acid gene sequencing. All cattle were fed a common corn-based finishing diet. Control cattle (CON) were administered no treatment while treated cattle (TRT) were supplemented daily with 0.050 g of MicroSaf 4C 40 (2 billion colony forming units of *Bacillus spp.*; Phileo by Lesaffre, Milwaukee, WI). Immediately after harvest and evisceration, the cecal contents of cattle were sampled. After DNA extraction, amplification, and sequencing, reads from CON samples ($N = 12$) and TRT samples ($N = 12$) were assigned taxonomy using the SILVA 138 database. Total morbidity, first treatment of atypical interstitial pneumonia, and early shipments for harvest were decreased among TRT cattle compared to CON cattle ($P \leq 0.021$). On average, cecal microbiota from TRT cattle had greater alpha diversity than microbiota from CON cattle as measured by Shannon diversity, Pielou's evenness, and feature richness ($P < 0.010$). Additionally, TRT microbial communities were different ($P = 0.001$) and less variable ($P < 0.001$) than CON microbial communities when evaluated by unweighted UniFrac distances. By relative abundance across all samples, the most prevalent phyla were Firmicutes (55.40%, SD = 15.97) and Bacteroidetes (28.17%, SD = 17.74) followed by Proteobacteria (6.75%, SD = 10.98), Spirochaetes (4.54%, SD = 4.85), and Euryarchaeota (1.77%, SD = 3.00). Spirochaetes relative abundance in TRT communities was greater than that in CON communities and was differentially abundant between treatments by ANCOM testing (W = 11); Monoglobaceae was the only family-level taxon identified as differentially abundant (W = 59; greater mean relative abundance in TRT group by 2.12 percentage points). Half ($N = 6$) of the CON samples clustered away from all other samples based on principal coordinates and represented cecal dysbiosis among CON cattle. The results of this study indicated that administering a four-species blend of *Bacillus* positively supported the cecal microbial communities of finishing cattle. Further research is needed to explore potential mechanisms of action of *Bacillus* DFM products in feedlot cattle.

Lay Summary

Microbes in the rumen break down fiber and complex nutrients into energy that cattle can absorb. Rumen microbes are becoming well studied, but the microbes of the hindgut—specifically of the cecum and large intestine—are less well-studied. As feedlot cattle eat large amounts of grain, maintaining health and balance of microbes in the hindgut is important. Overconsumption of a meal causes a greater proportion of digestion to occur in the hindgut, causing greater acid production that damages the gastrointestinal lining. If dietary microbial supplements support a more diverse microbial population, the challenges caused by greater hindgut digestion could be mitigated. To test this, cecal microbes were characterized after feedlot cattle were fed a conventional diet, with or without a supplement of *Bacillus* bacteria. Cecal samples from cattle that were fed *Bacillus* had greater microbial diversity. Approximately half of the cecal samples from cattle that were not fed *Bacillus* had disrupted microbial balance. Based on taxonomic assignment, bacteria observed in these disrupted samples indicated greater energy density of digesta and increased methane production. Supplementing feedlot cattle with *Bacillus* could improve hindgut microbial diversity.

Key words: *Bacillus*, cecum, feedlot, 16S ribosomal ribonucleic acid

Abbreviations: ASV, amplicon sequence variant; DFM, direct-fed microbial; EMP, Earth Microbiome Project; PCoA, principal coordinate analysis

Introduction

Bovine gastrointestinal microbes impact digestion, growth performance, and health of cattle (Myer, 2019). The relative abundance of microbial populations in the rumen has become well characterized (Hobson and Stewart, 1997; Petri et al., 2013; Henderson et al., 2015). However, less is known about the microbial communities of the hindgut. Divergence of microbial relative abundance has been demonstrated between the rumen and cecum (de Oliveira et al., 2013; Myer et al., 2015a; Bergmann, 2017). Additionally, grain feeding and acidosis have had greater impacts on the hindgut microbiota compared to the foregut microbiota (Khafipour et al., 2016). Dysbiosis of the hindgut has been identified as a factor contributing to overgrowth of organisms associated with negative health outcomes (Zeng et al., 2017; Simpson et al., 2018). For example, Clostridiaceae bacteria have been considered ubiquitous in the hindgut (Myer et al. 2015a, 2016; Freetly et al., 2020), yet *Clostridium perfringens* overgrowth has been associated with necrotic enteritis in growing and finishing cattle (Simpson

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et al., 2018; Yang et al., 2019). Direct-fed microbial (DFM) products have been investigated for modulating the rumen and fecal microbiota for potential health impacts (Krehbiel et al., 2003; McAllister et al., 2011; Elghandour et al., 2015). Proposed mechanisms of action of DFM products that could aid in preventing dysbiosis include competitive inhibition, immune stimulation, modulation of fermentation, and antimicrobial effects (McAllister et al., 2011). Few published studies have tested the effects of DFM products on cecal microbiota, especially in bovine models. The objective of this study was to evaluate the effects of a commercial *Bacillus* DFM product, MicroSaf 4C 40 (Phileo by Lesaffre, Milwaukee, WI), on the cecal microbiota of finishing cattle at the time of harvest.

Materials and Methods

Animal care and management protocols followed the recommendations of the *Guide for the Care and Use of Agricultural Animals in Research and Teaching, 4th Edition*. Since all cattle were harvested in a commercial processing facility and samples were collected postmortem, no IACUC approval was sought for this research.

Description of treatments, cattle management, and cattle selection

Commercial feedlot cattle from a single High Plains feedlot were used to evaluate the effect of *Bacillus* DFM supplementation on cattle health and postmortem cecal microbial communities. Cattle were managed in large pen conditions (170 ± 30.2 cattle per pen) and fed 153 d (SD = 12.2). After an adaptation period of 27 d, cattle were fed a high-concentrate basal diet (formulation and nutrient composition provided in Table 1). Cattle in the control group (CON) received no additional microbial feed additives. Cattle in the treated group (TRT) received diets formulated to provide each animal 0.050 g of a *Bacillus* supplement daily once acclimated to the finishing diet. The *Bacillus* supplement provided 2 billion colony forming units of a combination of *Bacillus amyloliquefaciens*, *B. subtilis*, *B. pumilus*, and *B. lichenformis* daily. Treatment was assigned based on pen location within the feedlot to minimize the effects of pen conditions. A total of 260 pens (42,495 cattle) were assigned to CON, and 51 pens (9,461 cattle) were assigned to TRT. Cattle assigned to TRT received *Bacillus* supplementation for no less than 90 d. Morbidity (total number of treatments, first treatments for atypical interstitial pneumonia, and first treatments for blot) and early shipments (cattle harvested before their home lot because of suspected metabolic disease) were recorded on an individual level and summarized as pen-level counts.

Table 1. Ingredient formulation and chemical composition of the finishing diet

Item	Value
Ingredient, % dry matter	
Steam flaked corn	43.13
High-moisture corn	33.89
Wet distillers grain	9.61
Corn silage	6.01
Supplement[1]	4.15
Corn oil	1.65
Mixed hay	1.56
Diet composition[2, 3]	
Dry matter, %	59.98
Crude protein, %	13.85
Non-protein nitrogen, %	1.01
NE_M, Mcal/kg	2.22
NE_G, Mcal/kg	1.53

[1]Supplement provided dietary concentration of 0.01691 g/kg of monensin sodium (Rumensin, Elanco, Greenfield, IN).
[2]All values except diet DM on a dry matter basis.
[3]Tylosin phosphate (Tylan, Elanco) was fed through the micromachine to provide each animal with 0.075 g daily.

At the time of harvest, a subset of cattle was selected to characterize cecal microbial populations. For postmortem cecal microbial sampling, the study population was defined as cattle that weighed between 350 and 450 kg and arrived at the feedlot over a 9-d period beginning on October 12, 2019 and included 408 cattle. At feedlot arrival, CON cattle weighed 384 kg (SD = 25.8) and TRT cattle weighed 407 kg (SD = 36.6). At harvest, 30 cattle (15 each from CON and TRT lots) were randomly selected for cecal microbial sampling.

Microbial sampling

Cattle were harvested on a single day at a High Plains commercial beef processing plant. Immediately after evisceration, the ceca of 15 cattle from CON and the ceca of 15 cattle from TRT groups were sampled for microbial analysis. Each cecum was opened, and digesta was sampled with sterile PurFlock Ultra Regular Tip Swab (Puritan Medical Products, Guilford, ME). Following sampling, swabs were immediately transported on ice to the Center for Meat Safety and Quality at Colorado State University (Fort Collins, CO). Samples were stored at −80 °C.

DNA extraction, amplification, and sequencing

Extraction of DNA and library preparation were performed at the Metcalf Laboratory at Colorado State University consistent with the recommendations of Weinroth et al. (2022). Manufacturers' protocols were used to extract DNA using the MO BIO MagAttract Powersoil DNA Extraction Kit (Qiagen, Carlsbad, CA) and a KingFisher Flex robot (Thermo Fisher Scientific, Waltham, MA). Cecal samples were loaded into a 96-well extraction plate by cutting the inoculated swab tip into the plate well with location randomly assigned to samples, uninoculated swabs ($N = 5$), negative controls ($N = 7$), and one positive control (ZymoBIOMICS Microbial Community Standard 6300; Zymo Research, Irvine, CA).

Amplicon library preparation was completed by polymerase chain reaction (PCR) with barcoded primers targeting the V4 region of the 16S rRNA gene. The barcode assay adapted for the Illumina MiSeq (Illumina; San Diego, CA) was used and included Illumina adaptor, barcode, spacer, and primer. Earth Microbiome Project (EMP) primers 515F and 806R were used for amplification (Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016). Duplicate PCR runs were conducted using an Eppendorf Vapo.Protect MasterCycler Pro-S thermocycler (Eppendorf, Hauppauge, NY). Conditions for PCR followed EMP protocols and included initial denaturation at 94 °C for 3 min; 30 cycles of denaturation

(94 °C, 45 s), annealing (50 °C, 60 s), and elongation (72 °C, 90 s); and a final 10-min extension at 72 °C (Gilbert et al. 2010, 2014; Thompson et al., 2017). Amplicons were subjected to agarose gel electrophoresis to visualize correct sizes of PCR products and the absence of signal from negative controls. Products were evaluated for effective amplification by agarose gel electrophoresis with expected band size of approximately 300 to 350 base pairs.

Concentration of amplicon products was determined by Quant-IT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) read on a Fluoroskan (Thermo Fisher Scientific) plate reader. Amplicons were pooled to form the sequencing library with a target inclusion of 300 ng of DNA from each sample. No more than 50 µL from a single sample was added to maintain the integrity of the negative control. Pooled amplicons were cleaned using MinElute PCR Purification Kit (Qiagen) following manufacturer protocols. Cleaned libraries were evaluated for amplicon concentration by NanoDrop Lite spectrophotometer (Thermo Fisher Scientific).

The amplicon library was diluted to a loading concentration of 8 pM and combined with 15% PhiX control library. Paired-end sequencing (2 × 250 bp) was performed using the 500 cycle MiSeq Reagent Kit v2 (Illumina) on the Illumina MiSeq platform at the Next Generation Sequencing Core Laboratory at Colorado State University.

Sequence processing

Amplicon sequence data were bioinformatically processed in QIIME2 version 2020.8 (Bolyen et al., 2019) using the High-Performance Computing Center at Texas Tech University. Barcodes, forward, and reverse sequences were imported, demultiplexed, and filtered for quality using the q2-demux plugin (Hamday et al., 2008; Hamday and Knight, 2009CJML_BIB_J_0029CJML_BIB_J_0028). Sequences were denoised with DADA2 (Callahan et al., 2016) with forward reads trimmed from 15 to 220 base pairs and reverse reads trimmed from 12 to 155 base pairs. Taxonomy was assigned to amplicon sequence variants (ASV) with the q2-feature-classifier plugin and classify-sklearn naive Bayes classifier (Pedregosa et al., 2011; Bokulich et al., 2018). Reference sequences specific to the V4 region from Silva 138 database (Pruesse et al., 2007; Quast et al., 2013) were used to identify ASV at a 99% similarity threshold.

The sequencing depth of each negative control was evaluated to ensure cleanliness of extraction and library preparation; the number of reads generated by each control well before and after denoising was recorded. The sequencing depth of the positive control was similarly recorded. Additionally, the taxa relative abundance of the positive control was exported and visualized in R version 4.1.3 (R Core Team, 2022) using the geom_bar function of ggplot2 (Wickham, 2009) and compared to the known composition of the mock community for qualitative confirmation.

Reads classified as mitochondria and chloroplasts were removed from the data set. Additionally, all reads generated by controls and technical replicates were removed. All reads from six samples that yielded less than 500 denoised sequences were also removed. Features observed in only one sample were disregarded for further analysis. ASV were assigned phylogeny using SEPP methodology to construct an insertion tree with q2-fragment-insertion (Matsen et al. 2010, 2012; Janssen et al., 2018). Adequate sampling depth was justified by constructing a rarefaction curve with alpha diversity metrics. Sampling depth was standardized for diversity analysis by subsampling without replacement (Weiss et al., 2017) to 23,389 sequences per sample using q2-diversity.

Classification of dysbiosis

Dysbiosis was classified based on rarefied abundance data exported from QIIME2 as relative abundance. For each microbial family observed in greater than 5% relative abundance across all samples, the mean relative abundance ± 1 SD was calculated. By family, each sample was compared the respective range of the mean ± 1 SD. If an individual sample's relative abundances were outside of this range for half or more of the family-level taxa, the microbial community was considered in a state of dysbiosis.

Statistical analysis

Health outcomes were analyzed by logistic regression of count data summarized by pen using R version 4.1.3 (R Core Team, 2022). Taxa differential abundance was evaluated using QIIME2 by ANCOM testing at both the phylum and family level from rarefied sequence counts (Mandal et al., 2015). Significance for differential abundance was evaluated as a W value indicating log-fold change against a model-determined threshold based on a bimodal distribution. Alpha diversity was measured by richness (the number of observed features), Pielou's evenness (Pielou, 1966), and Shannon diversity index (Shannon, 1948). Beta diversity was measured as unweighted UniFrac distances (Lozupone and Knight, 2005). Principal Coordinate Analysis (PCoA) was used to spatially visualize samples (Vázquez-Baeza et al., 2017). Microbial relative abundance, alpha diversity measures, unweighted UniFrac distance matrix, and principal coordinates were exported from QIIME2 and imported into R using the qiime2R package (Bisanz, 2018). Differences in alpha diversity were evaluated between treatment groups with Kruskal-Wallis testing (Kruskal and Wallis, 1952). K-means clustering was used to group samples (Lloyd, 1957; MacQueen, 1967). Treatment groups and clusters were evaluated for beta diversity with PERMANOVA testing (Anderson, 2017) using the vegan package of R (Oksanen et al., 2022). The individual cecal microbial community was considered the experimental unit. Statistical significance was established at $P < 0.050$. All data were visualized in R version 4.1.3 (R Core Team, 2022) using the geom_boxplot and geom_bar function of ggplot2 (Wickham, 2009).

Results

Health performance

Total morbidity, first treatment of atypical interstitial pneumonia, and early shipments for harvest were decreased among TRT cattle compared to CON cattle ($P \leq 0.021$; Table 2). No statistical differences were observed in morbidity during the last 60 d of the feeding period ($P \geq 0.208$).

Sequencing results

A total of 1,744,899 sequence reads were generated for cecal samples, technical replicates, and controls. Following denoising, 1,218,231 reads remained. Denoised sequencing depths of positive controls and cecal samples were almost three magnitudes of order greater than negative controls (Table 3). Of the eight bacteria represented by the positive control, seven were identified at the genus level, and the remaining

Table 2. Health outcomes of cattle

Treatment				
	CON[1]	TRT[2]	SEM[3]	*P*-value
Total lots, *N*	260	51		
Total cattle, *N*	42,495	9,461		
Morbidity, whole feeding period (%)				
Total	12.29	11.43	0.327	0.019
AIP[4], first treatment	0.17	0.07	0.028	0.021
Bloat, first treatment	0.14	0.18	0.044	0.391
Morbidity, last 60 DOF (%)				
Total	2.21	2.35	0.156	0.402
AIP	0.11	0.06	0.026	0.208
Bloat	0.09	0.13	0.037	0.242
Early shipments[5] (%)	0.08	0.01	0.013	0.007

[1]Cattle not administered MicroSaf 4C 40.
[2]Cattle administered MicroSaf 4C 40.
[3]Standard error of the mean.
[4]Atypical interstitial pneumonia.
[5]Cattle shipped for harvest prior to shipment of lot because of suspected metabolic disease.

Table 3. Mean sequence counts of samples and controls

	N	Sequences	Denoised sequences
Samples	24	56,257	48,732
Negative control, empty well	7	151	56
Negative control, swab[1]	5	550	54
Positive control[2]	1	73,696	64,549

[1]PurFlock Ultra Regular Tip Swabs (Puritan Medical Products, Guilford, ME).
[2]ZymoBIOMICS Microbial Community Standard 6300 (Zymo Research, Irvine, CA).

bacterium was identified at the family level. Once chloroplasts, mitochondria, features observed in only one sample, and controls were removed from the data set, samples from CON (N = 12) and TRT (N = 12) groups were retained representing 1,521 unique features with a mean sequencing depth of 39,229 (SD = 7,637). Only five sequencing reads classified as *Bacillus* were identified across all 24 samples retained in the data set.

Beta diversity

Based on PERMANOVA analysis of unweighted UniFrac distances, microbiota from cecal CON samples differed from microbiota of cecal TRT samples (Figure 1; P = 0.001, F = 4.235, permutations = 999). The phylogenetic distances among communities of CON cecal samples were greater than distances among communities of TRT cecal samples (Figure 2; $P < 0.001$, Kruskal-Wallis $\chi^2 = 56.32$). Greater distances between CON communities were caused by K-means cluster 2 which included six CON samples and segregated from K-means cluster 1 (which included 6 CON samples and all 12 TRT samples) by having a lesser value on the PCoA x-axis (Figure 1). All six samples in K-means cluster 2 were considered instances of dysbiosis; no samples in K-means cluster 1 were considered instances of dysbiosis (Supplementary Table S1).

Alpha diversity

Microbial diversity measured by Shannon Index ranged from 3.76 to 7.92. Mean Shannon Index for CON communities was 5.67 (SD = 1.33); mean Shannon diversity for TRT communities was 7.12 (SD = 0.40). On average, TRT microbial communities were 26% more diverse that CON microbial communities (P = 0.005; Figure 3). Shannon diversity differences were associated with TRT communities having both greater evenness as measured by Pielou's Index (P = 0.003; Figure 4) and greater richness as measured by number of observed features (P = 0.008; Figure 5). Within-group variation of Shannon diversity measured as SD was more than three-times greater for the CON group compared to TRT.

Variation between CON samples was caused by divergence of CON samples between K-means clusters. Mean Shannon Index for K-means cluster 1 communities was 6.93 (SD = 0.70); mean Shannon diversity for K-means cluster 2 communities was 4.78 (SD = 0.97). On average, K-means cluster 1 microbial communities were 45% more diverse that CON microbial communities (P = 0.001). Similarly, K-means cluster 1 had greater evenness and richness than K-means cluster 2 ($P \leq 0.001$). Richness was decreased more than 52% in K-means cluster 2 compared to that of K-means cluster 1. Mean number of observed features for K-means cluster 1 communities was 421 (SD = 68.6); mean number of observed features for K-means cluster 2 communities was 202 (SD = 52.3).

Phylum-level taxonomy

Classification of the sequence reads using the SILVA database identified five phyla in greater than 1.5% relative abundance across all samples. The two most prevalent phyla were Firmicutes (55.40% relative abundance, SD = 15.97) and Bacteroidetes (28.17% relative abundance, SD = 17.74). The ratio of Firmicutes to Bacteroidetes was calculated for each sample; mean Firmicutes to Bacteroidetes ratio was 157.45 (range: 0.58 to 1,207.11) for CON microbial communities and 1.51 (range: 0.66 to 2.02) for TRT microbial communities. No difference in Firmicutes to Bacteroidetes ratio was detected based on nonparametric Kruskal-Wallis testing (P = 0.119, H = 2.43).

Other phyla observed included Proteobacteria (6.75% relative abundance, SD = 10.98), Spirochaetes (4.54% relative abundance, SD = 4.85), and Euryarchaeota (1.77% relative abundance, SD = 3.00). The phylum Spirochaetes composed 2.00% (SD = 4.09) of CON microbial communities and 7.34% (SD = 4.25) of TRT communities and was identified as differentially abundant between treatments by ANCOM testing (W = 11).

Family-level taxonomy

Fifteen family-level taxa were observed in greater than 1.5% relative abundance across all reads (Table 4). These families are visualized in Figure 6. Of all the families observed, Monoglobaceae was the only family identified as differentially abundant by ANCOM testing (W = 59). The phylum Firmicutes was represented by Oscillospiraceae (10.89% relative abundance, SD = 5.96), Lachnospiraceae (9.81% relative abundance, SD = 8.70), Eubacterium coprostanoligenes group (6.57% relative abundance, SD = 8.44), Clostridiaceae

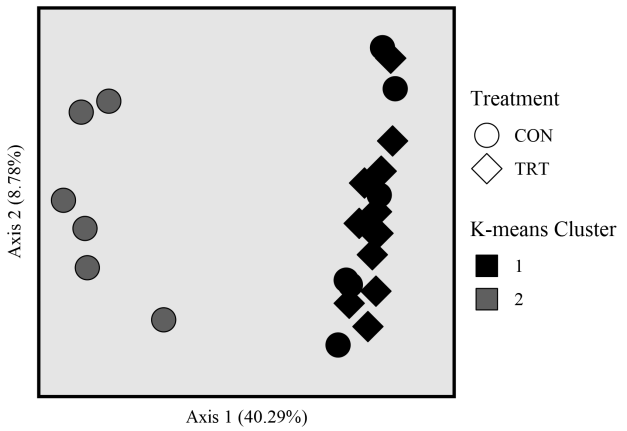

Figure 1. Principal coordinate analysis of cecal microbial communities from control cattle (CON, round marker) and cattle fed 0.050 g of MicroSaf 4C 40 daily (TRT, diamond marker). Microbiota of cecal CON samples differed from microbiota of cecal TRT samples based on PERMANOVA analysis of unweighted UniFrac distances ($P = 0.001$, $F = 4.235$, permutations = 999). Microbiota of cecal samples in K-means group 1 differed from microbiota of cecal samples in K-means group 2 based on PERMANOVA analysis of unweighted UniFrac distances ($P = 0.001$, $F = 14.275$, permutations = 999).

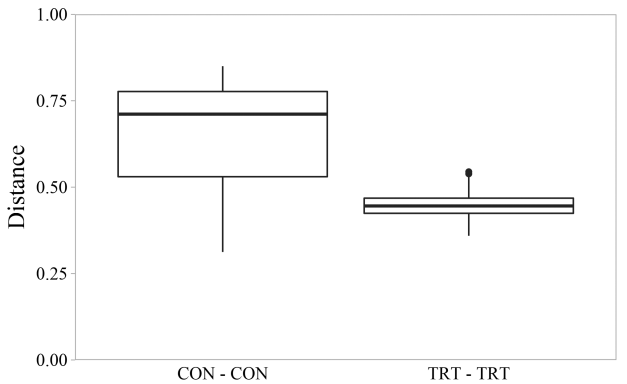

Figure 2. Pairwise distances between cecal microbial communities based on unweighted UniFrac distance matrix as evaluated by Kruskal–Wallis testing. Box plots represent distances between control cattle (CON) and distances between cattle fed 0.050 g of MicroSaf 4C 40 daily (TRT; supplementation of 2-billion colony forming units of a combination of four species of *Bacillus* daily). Distances between CON communities were greater than distances between TRT communities ($P < 0.001$, $\chi^2 = 56.32$, df = 1).

(5.63% relative abundance, SD = 8.26), Peptostreptococcaceae (4.44% relative abundance, SD = 5.01), Oscillospirales UCG-010 (3.11% relative abundance, SD = 3.41), Erysipelotrichaceae (2.27% relative abundance, SD = 3.45), and Monoglobaceae (1.66% relative abundance, SD = 1.64). The phylum Bacteroidetes was represented by the families Prevotellaceae (13.87% relative abundance, SD = 12.56), Rikenellaceae (6.43% relative abundance, SD = 4.92), Bacteroidaceae (5.08% relative abundance, SD = 3.65), and Muribaculaceae (1.94% relative abundance, SD = 1.65). The phylum Spirochaetes was represented by the Spirochaetaceae family (4.94% relative abundance, SD = 4.89) and the *Treponema* genus. The phylum Proteobacteria was represented by Succinivibrionaceae (5.03% relative abundance, SD = 11.95). The phylum Euryarchaeota was represented by Methanobacteriaceae (1.69% relative abundance, SD = 3.02). The genus

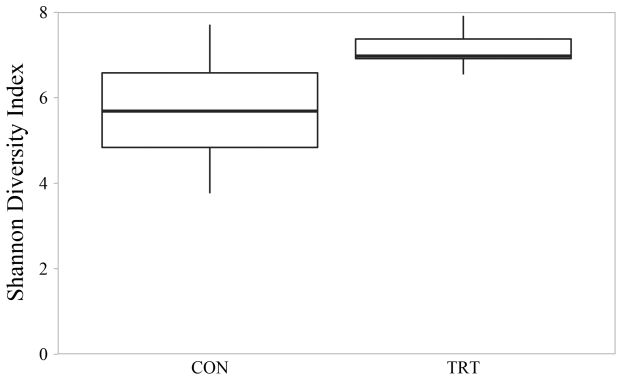

Figure 3. Shannon diversity index of cecal microbial communities of control cattle (CON) and cattle fed 0.050 g of MicroSaf 4C 40 daily (TRT; supplementation of 2-billion colony forming units of a combination of four species of *Bacillus* daily). Shannon diversity index differed between CON and TRT groups ($P = 0.005$, $H = 8.003$, $N = 24$).

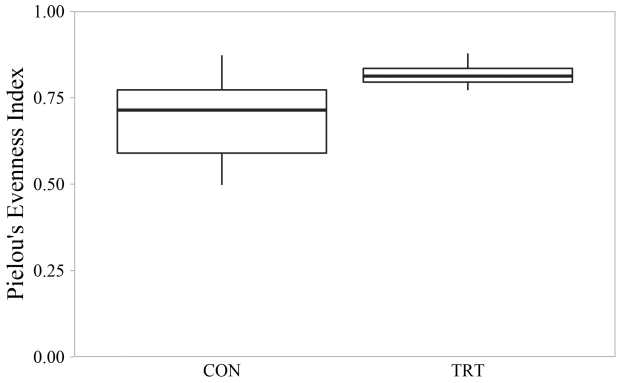

Figure 4. Evenness of cecal microbial communities measured by Pielou's Index of control cattle (CON) and cattle fed 0.050 g of MicroSaf 4C 40 daily (TRT; supplementation of 2-billion colony forming units of a combination of four species of *Bacillus* daily). Evenness differed between CON and TRT groups ($P = 0.003$, $H = 8.670$, $N = 24$).

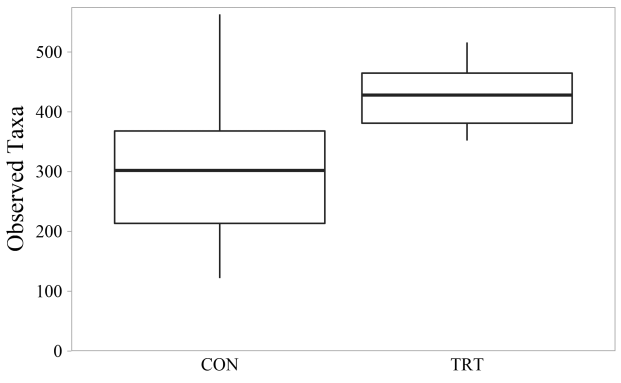

Figure 5. Richness of cecal microbial communities measured by the number of bacterial taxa observed of control cattle (CON) and cattle fed 0.050 g of MicroSaf 4C 40 daily (TRT; supplementation of 2-billion colony forming units of a combination of four species of *Bacillus* daily). Richness differed between CON and TRT groups ($P = 0.008$, $H = 7.056$, $N = 24$).

Methanobrevibacter composed the majority of all Methanobacteriaceae reads. For 13 of the 15 family-level taxa observed, the SD of CON relative abundances were greater than those of TRT relative abundances.

Table 4. Mean family-level taxa relative abundance of cecal microbial families observed in greater than 1.5% mean relative abundance across all reads

Relative abundance, % ± SD				
Phylum	**Family**	**Control[1] (*N* = 12)**	**Treated[2] (*N* = 12)**	**Treated:control**
Bacteroidetes	Prevotellaceae	11.55 ± 15.12	16.20 ± 9.45	1.40
	Rikenellaceae	5.20 ± 6.35	7.67 ± 2.64	1.47
	Bacteroidaceae	2.88 ± 3.18	7.28 ± 2.67	2.53
	Muribaculaceae	1.26 ± 1.52	2.63 ± 1.54	2.09
Firmicutes	Oscillospiraceae	7.66 ± 6.20	14.13 ± 3.59	1.85
	Lachnospiraceae	12.89 ± 11.50	6.72 ± 2.28	0.52
	Eubacterium coprostanoligenes group	8.93 ± 11.66	4.20 ± 0.85	0.47
	Clostridiaceae	7.00 ± 11.22	4.27 ± 3.59	0.61
	Peptostreptococcaceae	5.37 ± 6.72	3.52 ± 2.32	0.66
	Oscillospirales UCG-010	2.33 ± 3.74	3.89 ± 3.00	1.67
	Erysipelotrichaceae	3.16 ± 4.72	1.37 ± 0.93	0.43
	Monoglobaceae	0.60[b] ± 1.19	2.72[a] ± 1.33	4.53
Proteobacteria	Succinivibrionaceae	7.40 ± 16.22	2.67 ± 4.82	0.36
Spirochaetota	Spirochaetaceae	2.33 ± 4.14	7.55 ± 4.25	3.24
Euryarchaeota	Methanobacteriaceae	2.99 ± 3.91	0.40 ± 0.30	0.13

[1]Cecal microbiota of cattle not administered MicroSaf 4C 40.
[2]Cecal microbiota of cattle administered MicroSaf 4C 40.
[a,b]Family-level relative abundance differed by ANCOM testing.

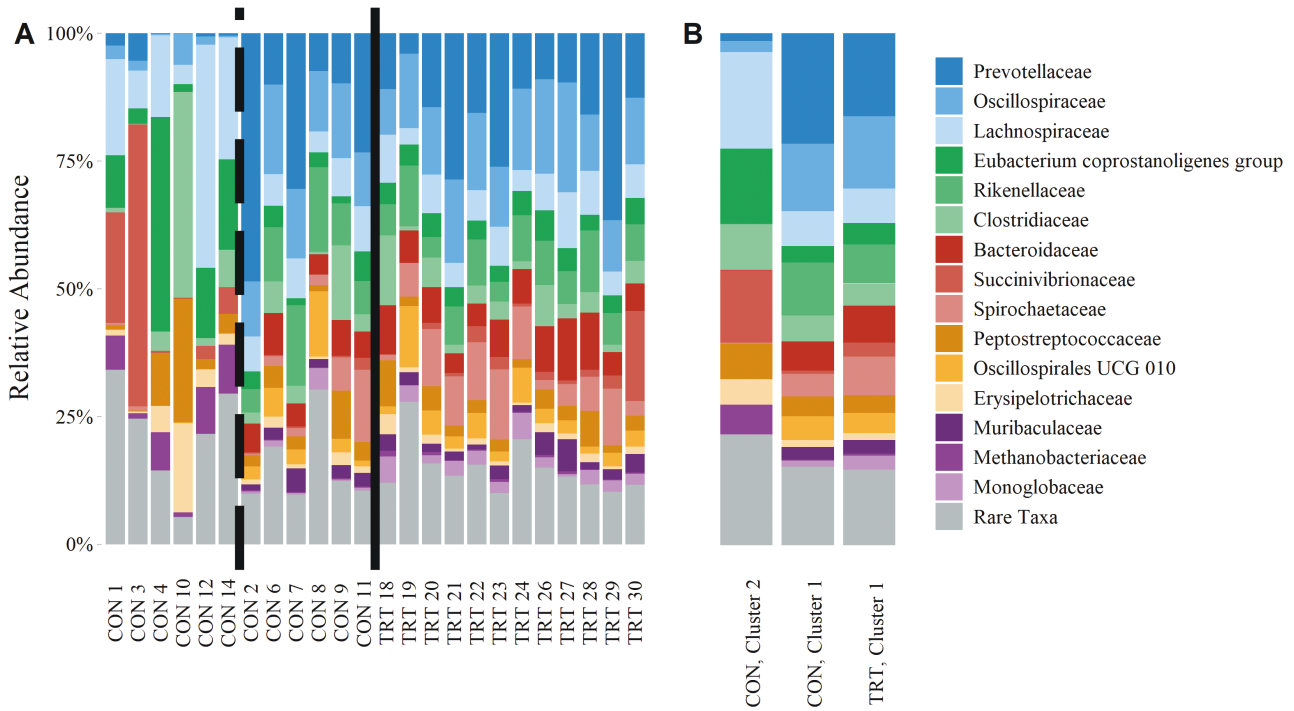

Figure 6. Relative abundance of family-level taxa composing the cecal microbial communities of control cattle (CON) and cattle fed 0.050 g of MicroSaf 4C 40 daily (TRT; supplementation of 2-billion colony forming units of a combination of four species of Bacillus daily). Rare taxa include all bacterial families observed in less than 1.5% average relative abundance across all samples. Microbial relative abundance by sample; solid line separates CON and TRT samples; the dashed line separates K-means clusters (A; as identified in Figure 1). Microbial relative abundance summarized by treatment and cluster (B).

Discussion

Diversity and dysbiosis

Microbial dysbiosis is related to pathologies of the gastrointestinal tract (Plaizier et al., 2014; Khafipour et al., 2016; Azad et al., 2019). Dysbiosis was observed in six CON samples that clustered away from all other samples (K-means cluster 2). Divergence of CON samples between K-means clusters drove greater variation in alpha diversity and beta diversity measures among CON samples (compared to TRT samples which all clustered together). The decreased variability between TRT microbial communities compared to that of CON communities was similar to the findings of Schofield et al. (2018) when *B. amyloliquefaciens* was fed to dairy calves and sheep. Together, these results suggested that feeding *Bacillus* could promote more consistent microbial communities between animals partly by decreasing the incidence of dysbiosis.

Overall, Shannon diversity values from the present study were intermediate to those identified within steer cecal microbiota by Freetly et al. (2020) and in steer colon microbiota by Myer et al. (2015a). Shannon values for the TRT group and K-means cluster 1 were similar to those previously reported from bovine fecal samples by Xu et al. (2014) and Durso et al. (2012). However, mean Shannon diversity value for K-means cluster 2 was approximately 30% less than previously reported values. This is further evidence that the communities in K-means cluster 2 were instances of dysbiosis. Decreased Shannon diversity values have been observed in the hindgut of cattle when dysbiosis coincided with a disease state (Fecteau et al., 2016). However, greater Shannon diversity of hindgut microbiota has been associated with improved feed efficiency (Welch et al., 2020). In models of other species, *Bacillus* spp. supplementation has increased intestinal alpha diversity and similarly resulted in improved daily weight gain and feed conversion (Sun et al., 2010; Hong et al., 2019; Li et al. 2019a, 2019b; Luo et al., 2020). Collectively, decreased alpha diversity in the CON communities represented in K-means cluster 2 was associated with dysbiosis; feeding *Bacillus* could mitigate instances of hindgut dysbiosis to improve digestive health and decrease morbidity and early shipments caused by metabolic disease.

Potential pathology

Clostridiaceae and related taxa were observed in numerically increased relative abundance in one sample of the current study. Control sample 10 (classified into K-means cluster 2) had 40.29% relative abundance of Clostridiaceae and 24.20% relative abundance of Peptostreptococcaceae. Clostridiaceae bacteria are known to be spore-forming bacteria that exist in greater relative abundance in the hindgut and contribute to digestion of carbohydrates and protein (Myer et al. 2015b, 2016; Freetly et al., 2020). However, the relative abundance observed in CON sample 10 could be approaching dominance of the microbial community. While 16S methodology does not measure viable cells, this finding could exemplify dysbiosis associated with a pathological state. *Clostridium sensu stricto 1*, the predominate genera of Clostridiaceae observed in this study, has been positively associated with *Clostridium perfringens* and *necrotic enteritis (*Yang et al., 2019). Peptostreptococcaceae is associated with the lumen of the hind gut (Mao et al., 2015). *Romboutsia*, the primary Peptostreptococcaceae genus observed in this study, is associated with *Escherichia coli O157:H7* challenge (Mir et al., 2019), and the second most observed Peptostreptococcaceae genus in this study, *Paeniclostridium*, is closely related to pathogenic *Clostridium* (Rabi et al., 2017). With greater than 60% of the microbial community of CON sample 10 composed of Clostridiaceae and Peptostreptococcaceae, this cecal sample exemplifies a state of dysbiosis with pathological implications.

Prevention of overgrowth of Clostridiaceae among TRT communities may have been modulated by the four species of *Bacillus* that were fed. *Bacillus* spp. have previously exhibited antimicrobial properties. *Bacillus subtilis* supplemented to Holstein cows supported hindgut health and reduced loads of *Clostridium* (Song et al., 2014). Protective properties of *Bacillus* spp. from the pathogens *Salmonella typhimurium* and *Escherichia coli* have also been demonstrated (Broadway et al., 2020; Lin et al., 2020). Secretion of the antimicrobial peptide subtilosin and the protease subtilin have been identified as products of *Bacillus subtilis* bacteria that enable antibacterial and antifungal properties (Algburi et al., 2020; Lin et al., 2020; Luise et al., 2022). As such, findings indicated that *Bacillus* supplementation could have protected TRT microbial communities from Clostridiaceae overgrowth.

Indications of hindgut nutrient availability

Feed intake, rate of passage, and microbial communities are interrelated (Colucci et al., 1982; Okine and Mathison, 1991CJML_BIB_J_0017CJML_BIB_J_0070; Freetly et al., 2020). After energy extraction has occurred in the rumen and small intestine, differences in cecal energy abundance could reflect rate of passage and extent of nutrient digestion. Numerically, CON sample 1 and 3 had greater abundance of Succinivibrionaceae. This bacterial family has been prevalent in the rumen and less prevalent in the hindgut because Succinivibrionaceae thrive when starch is available as a substrate (Hespell, 1992). The CON communities enriched in Succinivibrionaceae indicated that greater concentration of starch was reaching the hindgut. Consistent with poorer efficiency of starch use when digested in the hindgut compared to the foregut, Myer et al. (2015b) observed an inverse relationship between cecal relative abundance of Succinivibrionaceae and feed efficiency. Increased cecal Succinivibrionaceae populations indicated greater quantities of starch were available for digestion in the hindgut. In the two instances in which Succinivibrionaceae was increased in the cecal microbiota (CON 1 and 3), the community was also considered to be in dysbiosis, potentially caused by greater starch flow to the hindgut.

Similarly, numerically greater Erysipelotrichaceae relative abundance among CON microbial communities could have indicated greater energy density of digesta reaching the cecum and greater potential for cecal lipid digestion. The Erysipelotrichaceae family has been associated with lipid metabolism, energy density, and inflammation in human and animal models (Kaakoush, 2015; Minaya et al., 2020). Additionally, increased relative abundance of the Erysipelotrichaceae genus *Turicibacter* has previously been associated with feeding of high concentrate diets (Liu et al., 2014). Numerically greater Erysipelotrichaceae relative abundance among CON microbial communities indicated greater flow of digestible nutrients to the hindgut.

Along with suspected greater energy density of the digesta, CON communities observed in dysbiosis (K-means cluster 2) had numerically greater relative abundance of

Methanobacteriaceae. Specifically, the genus *Methanobrevibacter*, which is considered the primary methanogen of the hindgut, was numerically enriched in K-means cluster 2 samples (Kim and Whitman, 2014). Ramayo-Caldas et al. (2020) found a negative relationship between Succinivibrionaceae and methane, the product of Methanobacteriaceae fermentation. On the contrary, CON communities in the present study had greater mean relative abundance of both Succinivibrionaceae and Methanobacteriaceae bacteria compared to TRT microbiota. However, mean Succinivibrionaceae relative abundance is likely inflated among the CON group by samples 1 and 3 in which Succinivibrionaceae relative abundance was 21.6% and 55.2%. Consistent with the present study, Schofield et al. (2018) observed lesser *Methanobrevibacter* when ruminants were supplemented with *B. amyloliquefaciens*. Numerically decreased Methanobacteriaceae relative abundance among TRT samples indicated decreased hindgut methanogenesis and decreased nutrient utilization likely as a function of greater prececal extent of digestion.

Microbial abundances and fibrolytic families

Microbial relative abundances observed in this study were like previous results. Firmicutes and Bacteroidetes are commonly cited as the predominant phyla composing the gastrointestinal microbiota (Callaway et al., 2010; Durso et al., 2012; Liu et al., 2016). Proteobacteria, Spirochaetes, and Euryarchaeota have been reported as common phyla of the bovine digestive tract (Huebner et al., 2019; Andrade et al., 2020).

However, within the Firmicutes phylum and more specifically the Eubacteriales order, numerical trends in relative abundance of cellulolytic bacteria were observed. While CON communities had numerically greater Lachnospiraceae relative abundance, TRT communities had greater Monoglobaceae and numerically greater Oscillospiraceae and Oscillospirales UCG-010 relative abundance. Fibrolytic properties have been demonstrated for all these bacterial families. The Lachnospiraceae family has demonstrated butyrate production and cellulolytic activity (Cotta and Forster, 2006; Nyonyo et al., 2014; Bach et al., 2019). Monoglobaceae (which was solely represented by the genus *Monoglobus*, a pectinolytic bacterium) was also associated with capacity for fiber degradation (Kim et al., 2019). Oscillospiraceae, a basonym for Ruminococcaceae, has been identified as a cellulolytic family in ruminant gastrointestinal tracts (Deusch et al., 2017; Yoon et al., 2017). Oscillospiraceae has been identified in fecal microbiota of grazing beef cows when taxonomy was assigned using a SILVA database (Pruesse et al., 2007; Koester et al., 2020); others have identified Ruminococcaceae in the hindgut when taxonomy was assigned using Greengenes (DeSantis et al., 2006; Myer et al., 2015a; Freetly et al., 2020). Regardless, the relative abundance of Oscillospiraceae and Oscillospirales UCG-010 was likely associated with greater capacity for fiber degradation (Biddle et al., 2013).

Together, numerical differences in family relative abundance of taxa belonging to the Eubacteriales order between CON and TRT communities indicated redundancy in fibrolytic potential. The observed alteration of microbial taxa assumed to be digesting fiber in the cecum could have been modulated by the *Bacillus* treatment because *B. amyloliquefaciens* has demonstrated cellulase production (Lee et al., 2008; Sun et al., 2017). However, more targeted data on community function and enzyme presence is needed to determine if supplemental *Bacillus* has a meaningful impact on fibrolytic bacterial populations in the cecum of fed cattle.

Hindgut microbial communities and health

Improved richness and evenness of hindgut microbiota—such as that observed in the TRT microbial communities—could be associated with benefits to feedlot cattle health and growth performance (Gressley et al., 2011; Rodriguez-Jimenez et al., 2019; Sanz-Fernandez et al., 2020). As high-concentrate finishing diets are fed to feedlot cattle, risk of acidosis is increased (Owens et al., 1998). The US feeder and fed cattle supplies are influenced predominantly by Angus genetics because of premiums paid for black-hided cattle that yield well-marbled carcasses (Parish et al., 2012; Williams et al., 2012; McCabe et al., 2019). Angus genetics are associated with greater feed intake than that of other breeds (Retallick et al., 2017), and expected progeny differences for dry matter intake suggest that genetics within the Angus population will continue to increase feed intake (American Angus Association, 2022). Collectively, these factors have resulted in greater feed intake by feedlot cattle, which coincides with increased rate of passage and shifts a greater proportion of digestion to the hindgut (Church, 1988). As postruminal digestion increases, risk is increased for hindgut digestive challenges including acidosis, disruption of microbial communities, and overgrowth of pathogens.

The hindgut is suggested to be more vulnerable to acidic pH than the rumen. Absence of protozoal species in the hindgut has limited sequestration of rapidly fermentable carbohydrates (Hume, 1997) and no saliva secretions are observed (Erdman, 1988). Additionally, the linings of the rumen and hindgut are composed of different epithelial structures. The rumen epithelium is composed of four layers of cuboidal and squamous epithelium while the hindgut epithelium is composed of a monolayer of columnar epithelium covered by mucus (Church, 1988). In the event of acidotic stress, the barrier function of the gastrointestinal epithelium can be compromised and associated with leak of proinflammatory toxins such as lipopolysaccharide (Rodriguez-Jimenez et al., 2019). Since the intestinal content is highly immunogenic, Sanz-Fernandez et al. (2020) suggested that the local inflammation from the hindgut could greatly contribute to systemic inflammation. Widespread inflammation is a large energy cost (Sanz-Fernandez et al., 2020) and is implicated in disease complexes such as acute interstitial pneumonia (Loneragan and Gould, 2000). As such, prevention of hindgut acidosis and associated dysbiosis in feedlot cattle would positively affect cattle health and potentially growth performance.

Bacillus could have exhibited protection from gastrointestinal dysbiosis by modulating foregut fermentation and corresponding rate of passage. Interestingly, only five reads classified as *Bacillus* were observed in cecal communities in this study. This suggested that the primary mode of action of the *Bacillus* treatment was not as a dominant member of the cecal microbiota. *Bacillus* either enters vegetative growth at a more proximal location in the gastrointestinal tract (such as the rumen or small intestine), or its relative abundance was below the practical detectable limit of the sequencing depth of this analysis as demonstrated by Schofield et al.'s comparison of *Bacillus* recovery by qPCR and 16S sequencing (2018).

Implications

Results of this study indicated that cattle fed *Bacillus* exhibited less animal-to-animal variation of cecal microbiota compared to that of negative control cattle. No instances of cecal dysbiosis were observed in the microbial communities of cattle that were fed *Bacillus*, and greater alpha diversity was identified in TRT communities compared to CON communities. Under the management conditions of this study with a highly fermentable, high-moisture finishing diet, *Bacillus* protected against imbalances in the hindgut microbiota and improved cattle health. Future studies should evaluate rate of passage, extent of ruminal digestion, and culture confirmation of potentially pathogenic organisms. Additionally, well-replicated, large-pen studies should test differences in feed efficiency and growth performance.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of Interest Statement

Lynn D. Reed and Matt D. Cravey are employed by the manufacturer of the product used as a treatment in this study. All other authors declare no conflict of interest.

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