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Effects of choline chloride on the ruminal microbiome at 2 dietary neutral detergent fiber concentrations in continuous culture

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ABSTRACT

Our objective was to evaluate the effects of unprotected choline chloride (Cho) on the ruminal microbiome at 2 dietary neutral detergent fiber (NDF) concentrations. We hypothesized that the effects of Cho on ruminal bacterial populations would depend on NDF. Eight dual-flow continuous-culture fermentors were arranged in a duplicated 4 × 4 Latin square as a 2 × 2 factorial with the following treatments: (1) 30% NDF-control (30% NDF diet, no supplemental choline); (2) 30% NDF-Cho (30% NDF diet plus 1.9 g of choline ion per kg of dry matter); (3) 40% NDF-control (40% NDF diet, no supplemental choline); and (4) 40% NDF-Cho (40% NDF diet plus 1.9 g of choline ion per kg of dry matter). We did 4 fermentation periods of 10 d each and used the last 3 d for collection of samples of solid and liquid digesta effluents for DNA extraction. Overall, 32 solid and 32 liquid samples were analyzed by amplification of the V4 variable region of bacterial 16S rRNA. Data were analyzed with R (R Project for Statistical Computing) and SAS (SAS Institute Inc.) to determine effects of Cho, NDF, and NDF × Cho on taxa relative abundance. The correlation of propionate molar proportion with taxa relative abundance was also analyzed. At the phylum level, relative abundance of *Firmicutes* in the liquid fraction tended to be greater when Cho was supplemented with a 30% NDF diet. At the order level, Cho increased *Coriobacteriales* in solid fraction and decreased *Fibrobacterales* in liquid fraction. Moreover, Cho decreased abundance of *Clostridiales* and increased *Selenomonadales* in the solid fraction, only with the 30% NDF diet. For genera, lower abundance of *Pseudobutyrivibrio* resulted from Cho in solid and liquid fractions. Greater abundance of *Succinivibrio* in solid and *Selenomonas* and *Selenomonas 1* in liquid resulted from Cho with the 30% NDF diet. Propionate molar proportion was positively correlated with relative abundance of order *Selenomonadales* in solid and liquid fractions, and with genus *Succinivibrio* in solid and genera *Selenomonas* and *Selenomonas 1* in liquid. Our results indicate that Cho primarily decreases abundance of bacteria involved in fiber degradation and increases abundance of bacteria mainly involved in nonstructural carbohydrate degradation and synthesis of propionate, particularly when a diet with 30% NDF is provided.

Key words: propionate, ruminal bacteria, unprotected choline

INTRODUCTION

Choline is commonly supplemented to dairy cows in the form of rumen-protected choline chloride since it was discovered that the choline molecule is extensively degraded by ruminal microorganisms (Sharma and Erdman, 1989). Feeding rumen-protected choline chloride aims to supply choline for intestinal absorption and therefore improve metabolic status in the cow (Brusemeister and Sudekum, 2006; de Veth et al., 2016).

Early studies on choline supplementation to ruminants were performed with unprotected choline chloride evaluating a wide range of doses (0.5–3.0 g of choline ion per kg of DMI) either before discovering degradation of choline by ruminal microorganisms or feeding choline at high doses as an unsuccessful attempt to overwhelm its ruminal degradation. Such studies yielded inconclusive results regarding the effect of unprotected choline chloride on ruminal fermentation in finishing beef cattle (Swingle and Dyer, 1970; Rumsey, 1985) and lactating dairy cows (Erdman et al., 1984; Atkins et al., 1988). However, Neill et al. (1978) observed that ruminal microorganisms degraded choline into acetaldehyde and trimethylamine, the latter being further metabolized into methane. Moreover, accumulation of trimethylamine indicated incomplete metabolization

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of trimethylamine into methane, and therefore a possible saturation of the methyl-coenzyme M reductase enzymatic system, which may affect microbial ruminal fermentation.

Studies in humans have shown that a greater intestinal supply and degradation of choline into trimethylamine is associated with greater relative abundance of *Firmicutes* and lower relative abundance of *Bacteroidetes* (Cho et al., 2017). These are also the 2 predominant phyla of bacteria in the rumen (Kim et al., 2011; Salfer et al., 2018) whose abundances are influenced by the concentration of NDF in the diet. Similarly, greater relative abundance of *Firmicutes* and lower relative abundance of *Bacteroidetes* result from a reduction in dietary NDF concentration (Plaizier et al., 2017), which may therefore influence ruminal degradation of choline. Recently, Arce-Cordero et al. (2021) reported greater molar proportion of propionate and lower NDF digestibility in continuous culture when unprotected choline chloride was supplemented with 30% NDF diets; such effects were not observed with 40% NDF diets, indicating that utilization of choline by ruminal microorganisms is dependent on dietary NDF and may have an effect on propionate-synthesizing bacteria in the rumen. To our knowledge, the effects of unprotected choline on ruminal bacterial populations and their possible association with the type of diet have not been published.

Our objective was to evaluate the effects of unprotected choline chloride on the ruminal microbiome at 2 dietary NDF concentrations. Based on our previous results (Arce-Cordero et al., 2021), we hypothesized that unprotected choline chloride would differentially affect ruminal bacterial populations depending on dietary NDF concentration.

MATERIALS AND METHODS

Experimental Design and Diets

Procedures for care and handling of animals required for this experiment were approved by the Institutional Animal Care and Use Committee at the University of Florida. Details on the experimental design and diets can be found in our companion study (Arce-Cordero et al., 2021). Briefly, 8 fermentors of a dual-flow continuous culture system were used in a duplicated 4 × 4 Latin square design. Treatments were arranged in a 2 × 2 factorial with 2 concentrations of dietary NDF (30% and 40%) and 2 levels of unprotected choline chloride supplementation (0 and 1.9 g of choline ion per kg of diet DM). The following treatments were evaluated: (1) 30% NDF-control (30% NDF diet without supplemental choline), (2) 30% NDF-choline (30% NDF diet plus 1.9 g of choline ion per kg of DM), (3) 40% NDF-control (40% NDF diet without supplemental choline), and (4) 40% NDF-choline (40% NDF diet plus 1.9 g of choline ion per kg of DM).

Dietary ingredients and composition of experimental diets are shown in Table 1. Corn silage was dried for 72 h at 60°C in a forced-air oven (Heratherm, Thermo Scientific) and all ingredients were ground through a 2-mm screen in a Wiley mill (model no. 2; Arthur H. Thomas Co.). Additionally, one sample of each feed was ground through a 1-mm screen and used for chemical analyses.

The diet with 30% NDF was formulated based on NRC (2001) recommendations for a 680-kg Holstein cow producing 45 kg of milk (3.5% fat, 3.0% protein, and 4.8% lactose). The 40% NDF diet represents a greater NDF concentration expected to induce changes in ruminal fermentation and microbial populations in comparison to a 30% NDF diet, according to previous research (Plaizier et al., 2017).

The amount of supplemental choline is based on our companion study indicating greater propionate molar proportion and lower NDF digestibility in continuous-culture fermentors (Arce-Cordero et al., 2021) and it had been previously defined based on scientific literature. Because our goal was to determine the effects of unprotected choline on ruminal fermentation and microbiome, only literature in which unprotected choline was supplemented to ruminants was considered to determine our experimental dose. Although more recent and abundant literature on protected choline supplementation can be found, such studies had the objective of supplementing protected choline with the goal of supplying choline for intestinal absorption and further metabolic implications. Our experimental dose of unprotected choline chloride was chosen as an average from studies in which supplemental doses of choline ion of 0.9 g/kg of DM (Swingle and Dyer, 1970) and 3 g/kg of DM (Atkins et al., 1988) increased concentrations of VFA and bacterial counts in ruminal fluid, indicating a possible stimulatory effect of choline on ruminal microorganisms. Therefore, each fermentor was provided with 384 μL/d choline chloride (70% aqueous dilution), which expressed as choline ion, corresponds to 200 mg/d or 1.9 g/kg of DM. Treatments without choline supplementation were dosed with 384 μL/d distilled water.

Dual-Flow Continuous Culture System Operation

A dual-flow continuous culture system, originally described by Hoover et al. (1976) and recently described by Arce-Cordero et al. (2020) and Brandao et al. (2020), was used for this experiment. Fermentation process was controlled with constant agitation (100

Table 1. Ingredient and chemical composition of experimental diets

	Treatment[2]			
	30% NDF		40% NDF	
Item[1]	Ctrl	Cho	Ctrl	Cho
Ingredient				
Corn silage	42.0	42.0	25.0	25.0
Corn	20.5	20.5	15.6	15.6
Soybean meal	20.0	20.0	18.1	18.1
Grass hay	15.0	15.0	39	39
Calcium carbonate	1.0	1.0	0.8	0.8
Calcium phosphate	0.5	0.5	0.5	0.5
Mineral premix[3]	1.0	1.0	1.0	1.0
Choline chloride[4] (μL)	0	384	0	384
Chemical composition				
CP	16.9	16.9	16.9	16.9
Ether extract	3.36	3.36	2.65	2.65
NDF	30.0	30.0	41.9	41.9
ADF	16.8	16.8	23.6	23.6
Starch	30.6	30.6	21.3	21.3
NE_L[5] (Mcal/kg)	1.68	1.68	1.51	1.51

[1]Expressed as a percentage of DM unless otherwise stated.

[2]Experimental treatments resulting from the combination of 2 main factors. Concentration of NDF in the diet: 30% NDF and 40% NDF; and unprotected choline chloride supplementation: Ctrl (0 μL/d) and Cho (384 μL/d).

[3]Composition on a DM basis: 99% NaCl, 0.35% Zn, 0.2% Fe, 0.2% Mn, 0.03% Cu, 0.007% I, and 0.005% Co.

[4]70% aqueous solution of choline chloride.

[5]Estimated according to NRC (2001).

rpm), temperature at 39°C, and infusion of artificial saliva (Weller and Pilgrim, 1974) with 0.40 g/L urea, at 3.05 mL per minute to regulate liquid and solid passage rates separately at 11% h^{-1} and 5.5% h^{-1}, respectively. Anaerobic conditions were maintained with continuous infusion of N_2 gas using a N generator (Infinity XE 501X membrane nitrogen generator, Peak Scientific Instruments) to guarantee a constant flow of N_2 into the vessels.

Four fermentation periods of 10 d were carried out for this experiment. On the first day of each period, fermentors were inoculated with fresh ruminal contents collected from 2 cannulated Holstein cows (same 2 cows throughout the study) fed a TMR with 38% corn silage, 19% ground corn, 13% soybean meal, 11% cotton seed, 9% citrus pulp, 8.5% mineral premix, and 1.5% palmitic acid supplement (on a DM basis). Ruminal contents were collected approximately 1 h after morning feeding, strained through 2 layers of cheesecloth into prewarmed insulated jars, and immediately transported to the laboratory. At the laboratory, each fermentor was prewarmed and continuously flushed with N_2 gas at time of inoculation when it was filled with a 50:50 mix (vol/vol) of ruminal contents from both cows and cultured for 10 d.

Each fermentor was provided with 106 g/d DM of the corresponding diet (30% NDF or 40% NDF) distributed in 2 meals of 53 g of DM at 0800 and 2000 h. Due to choline's rapid rate of degradation by ruminal microorganisms (Neill et al., 1978), liquid choline chloride (70% aqueous dilution) was provided with a pipette by dividing the 384-μL daily dose into 4 equal aliquots of 96 μL that were dosed every 6 h (at 0200, 0800, 1400, and 2000 h) to simulate a slow release of choline through a semi-continuous supply during the day. Treatments without choline supplementation were dosed an equal volume of distilled water at the same time points.

Collection of Samples

The first 7 d of fermentation of each period were considered adaptation to experimental diets for stabilization of bacterial communities (Salfer et al., 2018). The last 3 d of each period were used for collection of data and samples. Effluent containers collecting solid and liquid digesta outflow were kept in an ice-cold water bath to prevent further microbial fermentation.

At the end of each day (24 h) of fermentation, total liquid and solid digesta effluents of the same fermentor were weighed, combined, and samples were collected and stored at −20°C for further analyses of propionate as detailed in Arce-Cordero et al. (2021). Briefly, 10 mL of sample was strained through 4 layers of cheese-

cloth, acidified with 100 μL of 50% H_2SO_4, and stored at −20°C for subsequent analysis.

Samples of bacteria for sequencing analysis were collected each sampling day from both liquid and solid effluent at 3, 6, and 9 h after morning feed provision. For the liquid effluent, 15 mL of liquid was collected at each time point, totaling 45 mL collected from the same fermentor each day. For the solid effluent, 200 mL was collected at each time point and strained through 4 layers of cheesecloth, totaling 25 g of solid sample collected from each fermentor per day. Immediately after collection both liquid and solid samples were stored at −80°C for subsequent DNA extraction.

Laboratory Analyses

Dietary feed ingredients (corn silage, grass hay, corn grain, and soybean meal) and freeze-dried samples of saliva and digesta effluent were analyzed for DM (AOAC, 1990; method 930.15), ash (AOAC, 1990; method 942.05), and total N (AOAC International, 2000; method 990.03) by rapid combustion with a micro elemental N analyzer (Vario Micro Cube, Elementar). Concentration of NDF (Van Soest et al., 1991) was analyzed in feed ingredients and digesta samples with heat-stable α-amylase and sodium sulfite adapted for Ankom[200] Fiber Analyzer (Ankom Technology).

Processing of samples for propionate analysis was described in Arce-Cordero et al. (2021). Briefly, samples were thawed at room temperature, centrifuged at 10,000 × *g* for 15 min at 4°C; the supernatant was mixed with a solution of crotonic acid and metaphosphoric acid and frozen overnight, thawed at ambient temperature, and then centrifuged at 10,000 × *g* for 15 min at 4°C. The final supernatant was mixed with ethyl acetate, vortexed, allowed to settle, and the top layer transferred to a chromatography injection vial. Concentrations of acetate and propionate in samples were determined by GC (Agilent 7820A GC, Agilent Technologies) with a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m 0.53 mm, Varian CP7767, Varian Analytical Instruments).

DNA Extraction

Samples were thawed at room temperature and combined across days and time points within the same period and fermentor, resulting in 32 samples of liquid effluent fraction and 32 samples of solid effluent fraction that were processed individually for analysis. Genomic DNA was extracted separately from liquid and solid effluent samples according to the methodology of Stevenson and Weimer (2007) and described by Dai et al. (2017) for samples of continuous-culture fermentors. For solid samples, 22 g was blended with extraction buffer consisting of Tris HCl, EDTA, and NaCl; then the blend was centrifuged at 500 × *g* for 15 min at 4°C. Supernatant obtained from solid samples and unprocessed liquid samples were processed equally; approximately 22 mL was centrifuged at 10,000 × *g* for 25 min at 4°C and the bacterial pellet obtained was resuspended in DNA extraction buffer.

Resuspended bacterial cells were processed in a Beadbeater machine (Biospec Products) with zirconium beads (BioSpec Products), 20% sodium lauryl sulfate solution, and phenol. The DNA was extracted through sequential centrifugations with phenol, phenol-chloroform, and chloroform; and precipitated with 3 *M* Na acetate buffer and isopropanol. After centrifugation with 70% ethanol the DNA pellet was resuspended in Tris-EDTA buffer. Following extraction, DNA concentration was measured with Qubit Fluorometer (Invitrogen) and stored at −80°C.

DNA Amplification and Sequencing

Amplification of the V4 variable region of bacterial 16S rRNA gene was performed according to Kozich et al. (2013) using dual-index primers (Caporaso et al., 2011). The PCR amplification reaction consisted of 1 μL of forward index primer (10 m*M*), 1 μL of reverse index primer (10 m*M*), 1 μL of DNA template (10 ng/μL), and 17 μL of Pfx AccuPrime master mix (Invitrogen). Reaction started with denaturation for 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and elongation for 5 min at 72°C. Amplicons were run on a 1% agarose gel to confirm success of the PCR, then normalized with a SequalPrep Normalization Plate Kit (Applied Biosystems), and used for construction of the DNA pool library. Overall, 32 samples of the liquid effluent fraction and 32 samples of the solid effluent fraction were sequenced at the University of Florida's Interdisciplinary Center for Biotechnology Research using a MiSeq reagent kit V2 (2 × 250 cycles run; Illumina) in an Illumina MiSeq platform (Illumina). The 16S rRNA gene amplicon sequencing data were deposited into the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) under accession number PRJNA798789.

Bacterial Sequence Data Analysis

Data obtained from 16S amplicon sequencing were analyzed with Quantitative Insights into Microbial Ecology version 2 (QIIME 2) pipeline (Bolyen et al., 2019). Paired-end raw reads were imported and quality of the initial bases was evaluated according to the

Interactive Quality Plot. Sequence quality control was performed with Divisive Amplicon Denoising Algorithm (DADA2) pipeline implemented in QIIME 2, including steps for filtering low-quality reads, denoising reads, merging paired-end reads, and removing chimeric reads. The align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin of QIIME 2 was used to generate the phylogenetic tree. Sequencing depth was normalized to 10,800 sequences per sample and the number of amplicon sequence variants, richness (Chao1), diversity (Shannon index), and Bray-Curtis distance were calculated by the core-metrics-phylogenetic method. Resulting amplicon sequence variants were classified into bacterial taxonomy (phylum, class, order, family, and genus) for both liquid and solid fraction samples using the q2-feature-classifier plugin of QIIME 2 and the SILVA 138 database (https://www.arb-silva.de/documentation/release-1381/). Only average relative abundances greater than 0.1% across all samples were considered for further analyses.

Statistical Analysis

Data analysis was performed using R (https://www.r-project.org/) and SAS 9.4 (SAS Institute Inc.). Results of bacterial community structure (Bray-Curtis distance) were analyzed using the vegan package of R (Callahan et al., 2016) and visualized by principal component analysis plots. The PERMANOVA test was implemented in QIIME 2 to determine the differences in community structure between dietary NDF concentrations (30% or 40%) and choline supplementation levels (control or supplemented with choline).

Data of relative abundance and α diversity were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc.). The model included the effects of dietary NDF concentration (NDF), unprotected choline chloride supplementation (Cho), and their interaction (NDF $\times$ Cho) as fixed effects, plus the random effects of period, square, and fermentor within square. Correlations between molar proportion of propionate and abundance of those bacterial taxa affected by either Cho or NDF $\times$ Cho were also analyzed in SAS using the Pearson CORR procedure. Threshold for significance was defined at $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ was considered a trend.

RESULTS AND DISCUSSION

A total of 4,524,223 raw reads were generated from 16S rRNA sequencing, which after filtering, denoising, merging, and removing chimeras using DADA2 pipeline, resulted in 2,064,581 high-quality sequences that were retained for analysis. Overall, 64 samples were sequenced, which 32 corresponding to solid fraction and 32 to liquid fraction. A total of 22 phyla, 36 classes, 69 orders, 150 families, and 341 genera were identified across samples of solid and liquid fractions.

The effects of Cho and NDF on bacterial community structure are presented in Figure 1. In both solid (panel A) and liquid (panel B) fractions, dietary NDF concentration affected bacterial community structure. As expected, a difference in dietary NDF concentration of approximately 10% (30% NDF vs. 40% NDF) affected the profile of bacterial communities, as has been reported previously in other studies (Plaizier et al., 2017) and confirms the suitability of our experimental diets to allow for proper evaluation of the effect of Cho under 2 contrasting scenarios established by different dietary NDF concentrations (NDF $\times$ Cho). Conversely, any effects of Cho on bacterial community structure of solid (panel C) or liquid (panel D) fractions were observed according to Bray-Curtis similarity index. However, as shown in Table 2 there was a trend to an interaction NDF $\times$ Cho on Chao 1 index in the solid fraction, indicating that Cho would increase richness with 40% NDF only. Additionally, Shannon index in the liquid fraction tended to be lower for the 30% NDF diet compared with 40% NDF. To better understand possible effects of Cho and NDF $\times$ Cho on bacterial communities of the rumen, we analyzed the effects of such factors on relative abundance at multiple taxonomic levels.

At the phylum level (Table 3), *Firmicutes* accounted on average for 39.7% of the sequences in the solid fraction, followed by *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, *Fibrobacteres*, and *Patescibacteria* (35.5%, 9.1%, 8.5%, 1.8%, and 1.2%, respectively). Abundance of phyla in the liquid fraction was dominated by *Bacteroidetes* (46.2% of sequences on average), whereas *Firmicutes*, *Proteobacteria*, *Spirochaetes*, *Patescibacteria*, and *Fibrobacteres* accounted on average for 25.8%, 12.2%, 8.2%, 1.3%, and 1.1%, respectively. Similar values of phylum relative abundances have been reported by Dai et al. (2019) and Monteiro et al. (2022) for samples collected from continuous-culture fermentors. We did not find any effect of Cho or NDF $\times$ Cho in the solid fraction; however, relative abundance of *Firmicutes* was greater for the 40% NDF diet, and conversely, *Proteobacteria*, *Spirochaetes*, and *Patescibacteria* were less abundant with the 40% NDF diet. In the liquid fraction, abundance of *Proteobacteria* and *Spirochaetes* was also lower, but *Bacteroidetes* were more abundant, when the 40% NDF diet was provided. In contrast with our results, Plaizier et al. (2017) reported a greater abundance of *Firmicutes* in ruminal fluid when diets with a lower NDF concentration were fed as an attempt to induce SARA in cows. However, McCann et al. (2016) analyzed liquid and solid samples of ruminal

Table 2. Effect of dietary NDF concentration and unprotected choline chloride supplementation on bacterial α diversity

	Treatment[1]					P-value[2]		
	30% NDF		40% NDF					
Item	Ctrl	Cho	Ctrl	Cho	SEM	NDF	Cho	NDF × Cho
Solid fraction								
Chao 1	546	533	468	582	49.1	0.69	0.18	0.10
Shannon	8.57	8.55	8.30	8.73	0.17	0.74	0.17	0.13
Liquid fraction								
Chao 1	482	475	519	505	32.5	0.12	0.62	0.88
Shannon	8.26	8.21	8.42	8.40	0.12	0.07	0.72	0.86

[1]Experimental treatments resulting from the combination of 2 main factors. Concentration of NDF in the diet: 30% NDF and 40% NDF; and unprotected choline chloride supplementation: Ctrl (0 g of choline ion per kg of DM) and Cho (1.9 g of choline ion per kg of DM).

[2]Significance of main effects of statistical model: concentration of NDF in the diet (NDF), supplementation of unprotected choline chloride (Cho), and interaction of concentration of NDF in the diet with unprotected choline chloride supplementation (NDF × Cho).

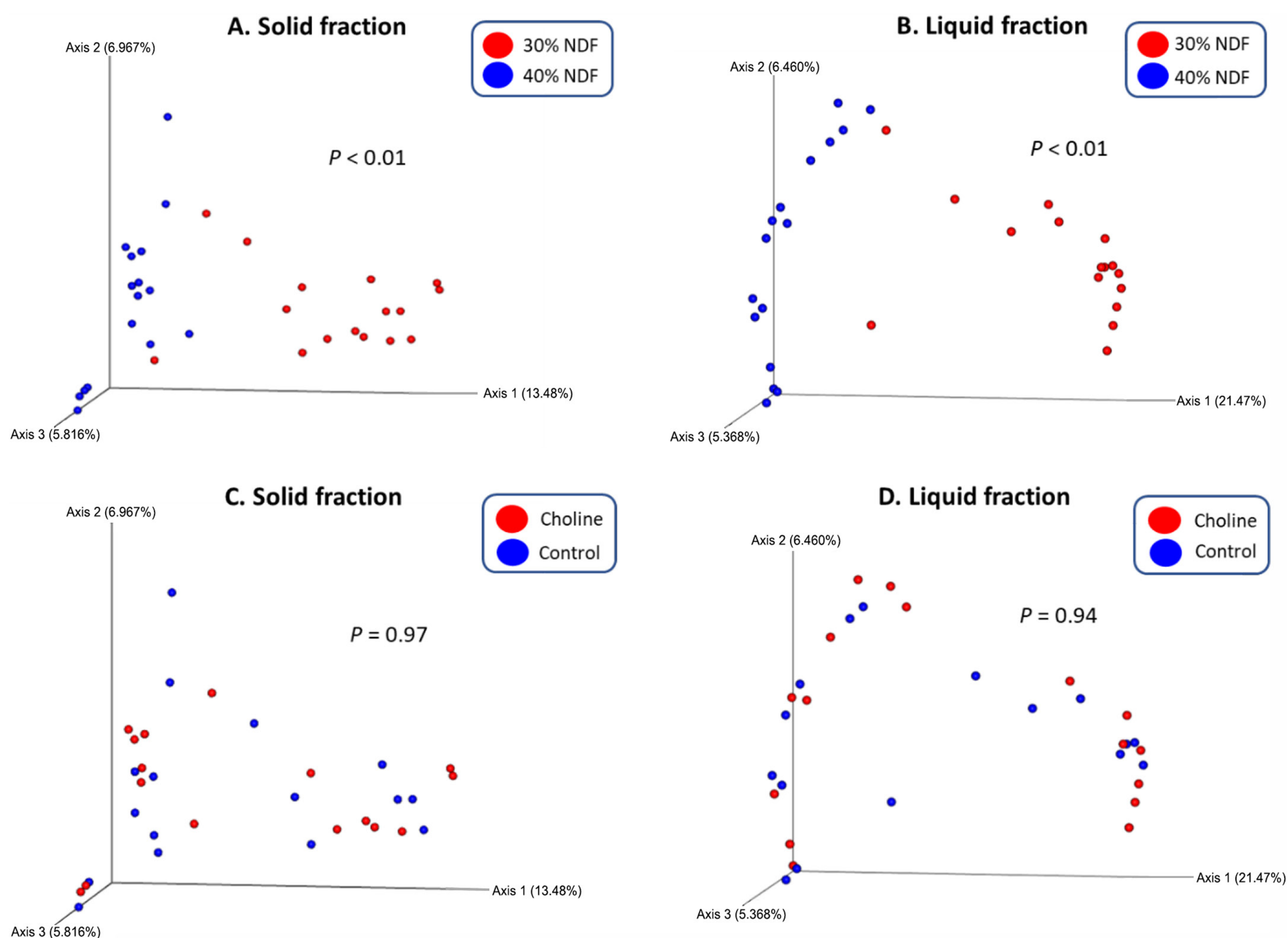

Figure 1. Principal coordinates analysis plots of Bray-Curtis similarity comparing the effects of dietary NDF concentration (panels A and B) and unprotected choline chloride supplementation (panels C and D) on community structure of ruminal bacteria. Dietary NDF concentrations evaluated were 30% NDF and 40% NDF, and unprotected choline chloride supplementation was evaluated at 0 (control) and 1.9 g (choline) of choline ion per kilogram of DM.

Table 3. Effect of dietary NDF concentration and unprotected choline chloride supplementation on relative abundance of main phyla in solid and liquid fractions

	Treatment[2]					P-value[3]		
	30% NDF		40% NDF					
Phylum[1]	Ctrl	Cho	Ctrl	Cho	SEM	NDF	Cho	NDF × Cho
Solid fraction								
Firmicutes	35.6	38.4	41.3	43.4	0.024	0.04	0.33	0.87
Bacteroidetes	35.8	34.6	36.2	35.7	0.017	0.56	0.49	0.75
Proteobacteria	12.0	11.1	7.25	5.98	0.018	<0.01	0.55	0.91
Spirochaetes	9.45	9.14	8.07	7.79	0.007	<0.01	0.53	0.97
Fibrobacteres	1.73	1.83	1.94	1.81	0.002	0.66	0.96	0.59
Patescibacteria	1.44	1.41	1.05	0.77	0.002	<0.01	0.31	0.43
Liquid fraction								
Bacteroidetes	42.5	43.2	48.4	50.5	0.031	<0.01	0.46	0.71
Firmicutes	25.5	28.9	25.9	23.1	0.030	0.14	0.88	0.09
Proteobacteria	16.0	12.0	9.79	10.9	0.017	0.04	0.40	0.14
Spirochaetes	9.82	8.62	7.19	6.99	0.010	<0.01	0.33	0.48
Fibrobacteres	0.83	1.30	1.21	1.03	0.002	0.75	0.30	0.03
Patescibacteria	1.53	1.13	1.25	1.17	0.002	0.41	0.10	0.26

[1]Taxonomic phyla with average relative abundance >1.0%.

[2]Experimental treatments resulting from the combination of 2 main factors. Concentration of NDF in the diet: 30% NDF and 40% NDF; and unprotected choline chloride supplementation: Ctrl (0 g of choline ion per kg of DM) and Cho (1.9 g of choline ion per kg of DM).

[3]Significance of main effects of statistical model: concentration of NDF in the diet (NDF), supplementation of unprotected choline chloride (Cho), and interaction of concentration of NDF in the diet with unprotected choline chloride supplementation (NDF × Cho).

contents, finding no effect of SARA on abundance of *Bacteroidetes* and *Firmicutes* in liquid samples, and a lower relative abundance of *Firmicutes* in the solid fraction under acidotic ruminal conditions. Similarly, Mao et al. (2013) reported a lower relative abundance of *Bacteroidetes* in ruminal fluid of cows experiencing SARA. Although we did not evaluate SARA as part of our experiment, certainly our 30% NDF diet was more similar to acidogenic diets than was our 40% NDF diet.

Effects of Cho on phylum abundance were limited. Relative abundance of *Firmicutes* in the liquid fraction tended to be greater when Cho was supplemented with the 30% NDF diet, indicating an important interaction NDF × Cho. To the best of our knowledge, no previous reports are available on the effects of unprotected choline chloride on the ruminal microbiome; however, Cho et al. (2017) also reported a greater abundance of *Firmicutes* in the intestine of humans consuming diets with a greater concentration of choline.

Effect of Cho was also observed as a trend toward decreasing the relative abundance of *Patescibacteria* in the liquid fraction, and an interaction NDF × Cho for abundance of *Fibrobacteres* in the same fraction. Although we are not aware of any reports on choline chloride effect on abundance of these phyla, Qiu et al. (2020) reported a decrease in abundance of *Patescibacteria* in the rumen of Holstein steers fed diets with a lower NDF concentration when evaluating a range between 29 and 48% NDF; such changes in ruminal microbiome were accompanied by an increase in ruminal propionate, which was also observed in our companion study as a consequence of Cho supplementation (Arce-Cordero et al., 2021).

Results on relative abundances of taxonomic orders in solid and liquid fractions are summarized in Tables 4 and 5, respectively. With the exception of 7 orders in the solid fraction and 5 orders in the liquid fraction whose abundances were not influenced by NDF, all the orders with an average relative abundance >0.1% were influenced by NDF. Our results show that compared with the 40% NDF diet, the diet with 30% NDF decreased the relative abundance of *Clostridiales* and conversely increased abundance of *Spirochaetales*, *Aeromonadales*, and *Selenomonadales* in both fractions analyzed. A correlation between bacteria within the order *Clostridiales* and milk-fat yield has been reported in dairy cows (Jami et al., 2014), suggesting a role of such bacteria on fiber fermentation and acetate synthesis in the rumen; therefore, it could be expected that the 40% NDF diet evaluated in our experiment represented a more favorable set of conditions for an increase in relative abundance of *Clostridiales*. Milk-fat synthesis is a complex process also involving hydrogenation and isomerization of fatty acids in the rumen; however, ruminal degradation of fiber plays a crucial role in which *Clostridiales* may be involved. This idea is supported by Söllinger et al. (2018) findings through meta-transcriptomics analysis, which revealed that *Clostridiales* is one of the key fiber-degrading bacterial groups in the rumen, encoding an abundant share of the cellulase and hemicellulose

Table 4. Effect of dietary NDF concentration and unprotected choline chloride supplementation on relative abundance of orders in solid fraction

Order[1]	Treatment[2] 30% NDF Ctrl	30% NDF Cho	40% NDF Ctrl	40% NDF Cho	SEM	*P*-value[3] NDF	Cho	NDF × Cho
Clostridiales	33.53	31.15	39.18	41.26	1.00	<0.01	0.97	0.04
Bacteroidales	34.17	34.29	35.51	34.07	1.00	0.34	0.28	0.22
Spirochaetales	9.29	9.04	8.01	7.68	0.72	<0.01	0.57	0.97
Aeromonadales	9.46	10.59	3.76	4.09	1.20	<0.01	0.37	0.68
Selenomonadales	5.96	7.31	4.37	4.19	0.60	<0.01	0.08	0.04
Fibrobacterales	1.88	1.81	2.18	1.89	0.20	0.24	0.27	0.48
Coriobacteriales	0.73	0.88	0.82	0.97	0.09	0.26	0.05	0.98
Absconditabacteriales	0.78	0.73	0.60	0.53	0.10	0.06	0.59	0.98
WCHB141	0.61	0.50	0.68	0.75	0.10	0.05	0.78	0.24
Mollicutes	0.54	0.46	0.49	0.50	0.13	0.92	0.56	0.51
Pirellulales	0.21	0.17	0.73	0.74	0.20	<0.01	0.80	0.85
Saccharimonadales	0.58	0.64	0.32	0.20	0.07	<0.01	0.61	0.17
Desulfovibrionales	0.39	0.37	0.36	0.36	0.06	0.67	0.87	0.73
Synergistales	0.32	0.30	0.30	0.30	0.05	0.96	0.66	0.96
Rickettsiales	0.08	0.08	0.53	0.34	0.06	<0.01	0.11	0.13
Izimaplasmatales	0.12	0.09	0.35	0.36	0.04	<0.01	0.84	0.58
Bacteroidia	0.23	0.28	0.12	0.09	0.04	<0.01	0.85	0.32
Erysipelotrichales	0.07	0.06	0.18	0.14	0.03	<0.01	0.15	0.33
Gastranaerophilales	0.12	0.19	0.04	0.05	0.04	<0.01	0.26	0.26
Burkholderiales	0.07	0.05	0.08	0.09	0.02	0.12	0.66	0.23
Bifidobacteriales	0.09	0.14	0.00	0.00	0.04	<0.01	0.54	0.59

[1]Taxonomic orders with average relative abundance >0.1%.

[2]Experimental treatments resulting from the combination of 2 main factors. Concentration of NDF in the diet: 30% NDF and 40% NDF; and unprotected choline chloride supplementation: Ctrl (0 g of choline ion per kg of DM) and Cho (1.9 g of choline ion per kg of DM).

[3]Significance of main effects of statistical model: concentration of NDF in the diet (NDF), supplementation of unprotected choline chloride (Cho), and interaction of concentration of NDF in the diet with unprotected choline chloride supplementation (NDF × Cho).

transcripts. Conversely, members of the order *Selenomonadales* have the ability to synthesize propionate through a succinate decarboxylation pathway (Paynter and Elsden, 1970) and therefore have been positively associated with ruminal concentration of propionate in dairy cows (Guyader et al., 2015; Popova et al., 2019).

Regarding the effects of Cho on relative abundance of orders, we observed that Cho increased *Coriobacteriales* in solid fraction and decreased *Fibrobacterales* and *Rickettsiales* in liquid fraction regardless of NDF level. A reduction in relative abundance of *Rickettsiales* has been reported in cows with SARA (Plaizier et al., 2017), and bacteria of the order *Fibrobacter* have been demonstrated to play a key role in synthesis of cellulase (Söllinger et al., 2018); therefore, a reduction in relative abundance of such orders resulting from Cho indicates an effect on ruminal bacteria involved in cellulose degradation. Considering that a constant supply of cellulose substrate was provided across treatments and that ruminal degradation of substrates is modeled as a first-order process (Waldo et al., 1972), the effect of choline on relative abundance of cellulolytic bacteria may be indirect, as a consequence of a direct stimulatory effect of choline on other taxonomic groups of bacteria associated with degradation of nonstructural carbohydrates.

An interaction between NDF × Cho was observed for orders in solid fraction, in which Cho decreased *Clostridiales* and increased *Selenomonadales* when supplemented with the 30% NDF diet, whereas in the liquid fraction NDF × Cho indicates an increase in *Selenomonadales* and a decrease in *Coriobacteriales* only with the 30% NDF diet. Lower relative abundance of *Clostridiales* and greater abundance of *Selenomonadales* have also been reported in dairy cows supplemented with 3-nitrooxypropanol, and a decrease in acetate molar proportion and greater molar proportion of propionate were observed in ruminal fluid (Lopes et al., 2016). Results of our companion study (Arce-Cordero et al., 2021) show that molar proportion of acetate decreased regardless of NDF; however, molar proportions of propionate and butyrate were greater only when Cho was supplemented with the 30% NDF diet (NDF × Cho), which is consistent with the NDF × Cho found for relative abundance of *Selenomonadales* in the present study. Our findings indicate that Cho increases relative abundance of *Selenomonadales* in the presence of an adequate supply of nonstructural carbohydrates, such as that provided with the 30% NDF diet. Average relative abundance of *Selenomonadales* in the present study averaged 5.5% (Tables 4 and 5), indicating that a substantial fraction of the bacterial

community was influenced by Cho supplementation, and thus could measurably affect production of propionate even though this acid is produced by multiple bacterial species.

Data corresponding to relative abundance of genera in both liquid and solid fractions are presented in Figure 2. Because the objective of our study was to evaluate the effect of Cho on ruminal bacteria and its possible interaction with NDF, for our analysis at the genus level we considered only the genera for which relative abundances were affected by either Cho or NDF × Cho. In the solid fraction (Figure 2, panel A), Cho decreased relative abundance of *Pseudobutyrivibrio*, *Prevotellaceae Ga6A1* group, and *Eubacterium coprostanoligenes* group, and conversely increased relative abundance of *Prevotella 7*, *Ruminococcaceae UCG002*, *Mogibacterium*, and *Clostridium sensu stricto 1*, regardless of NDF.

Interactions NDF × Cho were observed in the solid fraction for *Butyrivibrio 2*, *Ruminococcus 1*, *Eubacterium ruminantium* group, *Lachnospiraceae NK4A136* group, *Lachnospiraceae FCS020* group, *Christensenellaceae R-7* group, *Lachnospiraceae AC2044* group, *Lachnoclostridium 10*, and *Papillibacter* whose relative abundance decreased only when choline was supplemented with the 30% NDF diet. Moreover, the relative abundance of *Prevotellaceae YAB2003* group, *Succinivibrio*, and *Candidatus* Saccharimonas was increased by Cho only when the 30% NDF diet was provided.

Changes in genera relative abundance in the liquid fraction (Figure 2, panel B) indicate that a lower relative abundance due to Cho was observed for genera *Pseudobutyrivibrio*, *Veillonellaceae UCG001*, *Eubacterium coprostanoligenes* group, *Lachnospiraceae FCS020* group, and *XBB1006*. Conversely, relative abundance of *Prevotellaceae YAB2003* was increased by Cho. Regarding interactions NDF × Cho at the genus level, we observed that relative abundance of *Prevotella 1*, *DNF00809*, and *Termite treponema* cluster decreased when Cho was supplemented with the 30% NDF diet. Conversely, a greater relative abundance was observed in *Selenomonas*, *FD2005*, *Lachnospiraceae NK3A20* group, and *Selenomonas 1* when Cho was supplemented with the 30% NDF diet.

Our results on genera relative abundance are consistent with those observed for other taxa. Genera *AC2004*, *NK4A136*, and *FCS020* in the solid fraction and *FCS020* in the liquid fraction, all of them belonging to the *Lachnospiraceae* family, and *Christensenellaceae R-7* group in the solid fraction, responded to

Table 5. Effect of dietary NDF concentration and unprotected choline chloride supplementation on relative abundance of orders in liquid fraction

	Treatment[2]					P-value[3]		
	30% NDF		40% NDF					
Order[1]	Ctrl	Cho	Ctrl	Cho	SEM	NDF	Cho	NDF × Cho
Bacteroidales	42.86	41.21	51.35	51.68	2.00	<0.01	0.57	0.40
Clostridiales	16.45	15.55	20.12	19.65	0.80	<0.01	0.40	0.79
Aeromonadales	15.20	17.43	7.33	7.51	2.00	<0.01	0.32	0.39
Spirochaetales	10.00	10.03	6.23	6.53	0.80	<0.01	0.75	0.80
Selenomonadales	6.84	8.41	3.27	3.06	0.60	<0.01	0.21	0.10
Gastranaerophilales	1.36	1.50	1.00	1.07	0.40	0.11	0.67	0.88
Fibrobacterales	0.86	0.64	1.34	1.19	0.10	<0.01	0.05	0.70
WCHB141	0.71	0.42	1.27	1.39	0.20	<0.01	0.55	0.13
Absconditabacteriales	1.08	0.77	0.89	0.90	0.20	0.81	0.22	0.20
Izimaplasmatales	0.30	0.15	0.82	0.87	0.08	<0.01	0.51	0.17
Pirellulales	0.21	0.11	0.70	0.67	0.20	<0.01	0.49	0.73
Saccharimonadales	0.57	0.58	0.27	0.19	0.08	<0.01	0.64	0.58
Coriobacteriales	0.36	0.29	0.36	0.47	0.08	0.02	0.56	0.02
Desulfovibrionales	0.29	0.26	0.45	0.46	0.05	<0.01	0.70	0.33
Erysipelotrichales	0.32	0.36	0.33	0.23	0.07	0.26	0.56	0.22
Synergistales	0.19	0.15	0.36	0.30	0.05	<0.01	0.34	0.84
Mollicutes	0.32	0.18	0.27	0.22	0.07	0.94	0.11	0.49
Rickettsiales	0.09	0.03	0.43	0.30	0.06	<0.01	0.04	0.36
Bacteroidia	0.17	0.30	0.20	0.17	0.07	0.39	0.36	0.16
Burkholderiales	0.06	0.05	0.28	0.35	0.06	<0.01	0.58	0.45
Bifidobacteriales	0.24	0.32	0.00	0.00	0.09	<0.01	0.65	0.65

[1]Taxonomic orders with average relative abundance >0.1%.

[2]Experimental treatments resulting from the combination of 2 main factors. Concentration of NDF in the diet: 30% NDF and 40% NDF; and unprotected choline chloride supplementation: Ctrl (0 g of choline ion per kg of DM) and Cho (1.9 g of choline ion per kg of DM).

[3]Significance of main effects of statistical model: concentration of NDF in the diet (NDF), supplementation of unprotected choline chloride (Cho), and interaction of concentration of NDF in the diet with unprotected choline chloride supplementation (NDF × Cho).

Cho with a decrease in relative abundance that was only observed when the 30% NDF diet was provided. All of these genera belong to the order *Clostridiales*, where members of the *Lachnospiraceae* family are pectin-fermenting bacteria (Cotta and Forster, 2006) and *Christensenellaceae* family is associated with fermentation of structural carbohydrates (Morotomi et al., 2012). Relative abundances of both *Lachnospiraceae* and *Christensenellaceae* may also decrease after calving as a consequence of the dietary changes involving a greater intake of nonstructural carbohydrates (Bach et al., 2019). Our results indicate that Cho may affect some bacteria taxa involved in degradation of fiber, particularly when a 30% NDF diet is provided, in comparison to a 40% NDF diet.

Conversely, bacterial groups classified as members of the genus *Prevotellaceae* play a role in the synthesis of starch-degrading enzymes in the rumen (Söllinger et al., 2018). Similarly, greater relative abundance of bacteria of the genus *Succinivibrio* has been reported during SARA as a result of a greater supply of starch in the rumen (Plaizier et al., 2017). Moreover, an increase in abundance of *Selenomonas* observed in the liquid fraction when Cho is supplemented with the 30% NDF diet is consistent with the NDF × Cho interaction observed for the order *Selenomonadales*. Bacteria in genus *Selenomonas* are members of the order *Selenomonadales*; therefore, the increase observed in abundance of *Selenomonadales* could be explained by a greater abundance of *Selenomonas* when Cho is supplemented with the 30% NDF diet.

Given the consistency between our results of bacterial relative abundance in the present study and molar proportion of propionate from our companion study (Arce-Cordero et al., 2021) in response to Cho, we performed a Pearson correlation analysis between propionate

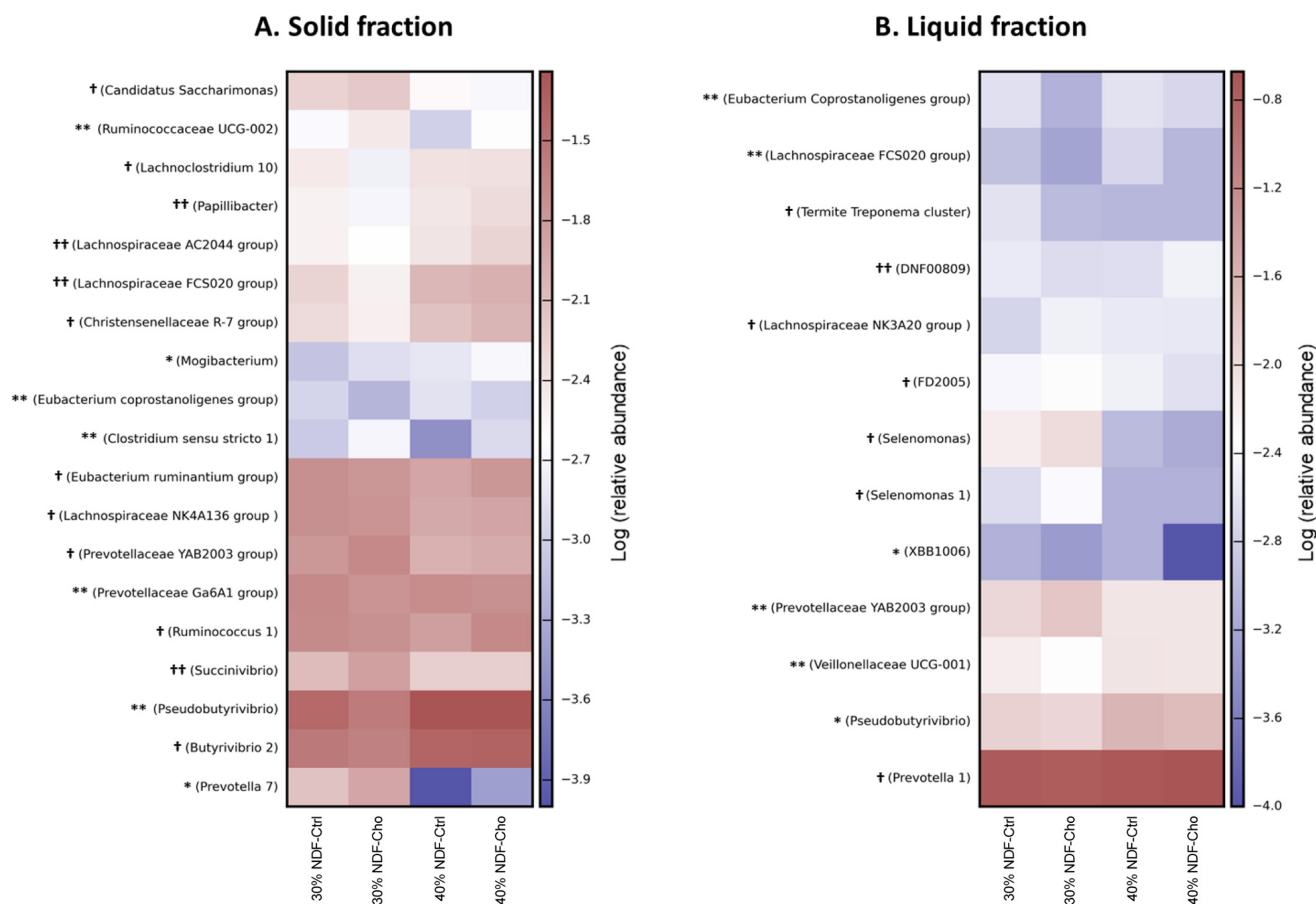

Figure 2. Relative abundance of genera that were influenced by unprotected choline chloride supplementation (Cho) or interaction between dietary NDF concentration and unprotected choline chloride supplementation (NDF × Cho). The effect of Cho is indicated by $**P \le 0.05$ and $*P \le 0.10$, and the effect of NDF × Cho is indicated by $††P \le 0.05$ and $†P \le 0.10$. Treatments are denoted as follows: (1) 30% NDF-Ctrl (30% NDF diet without supplemental choline), (2) 30% NDF-Cho (30% NDF diet plus 1.9 g of choline ion per kg of DM), (3) 40% NDF-Ctrl (40% NDF diet without supplemental choline), and (4) 40% NDF-Cho (40% NDF diet plus 1.9 g of choline ion per kg of DM).

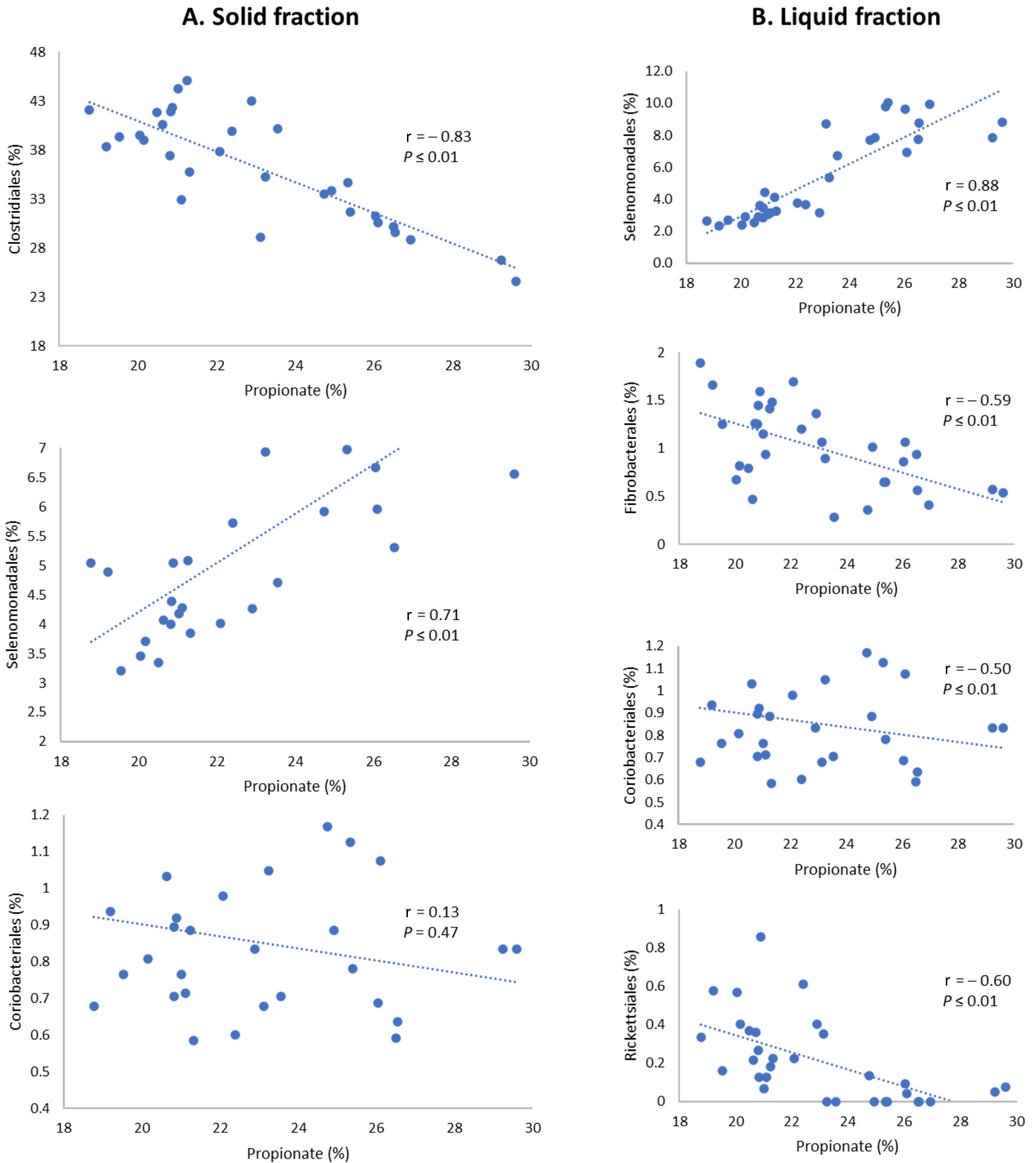

Figure 3. Correlation between molar proportion of propionate in ruminal fluid and relative abundance of orders in solid and fluid fractions. Only orders influenced by unprotected choline chloride supplementation (Cho) or its interaction with dietary NDF concentration (NDF × Cho) were analyzed.

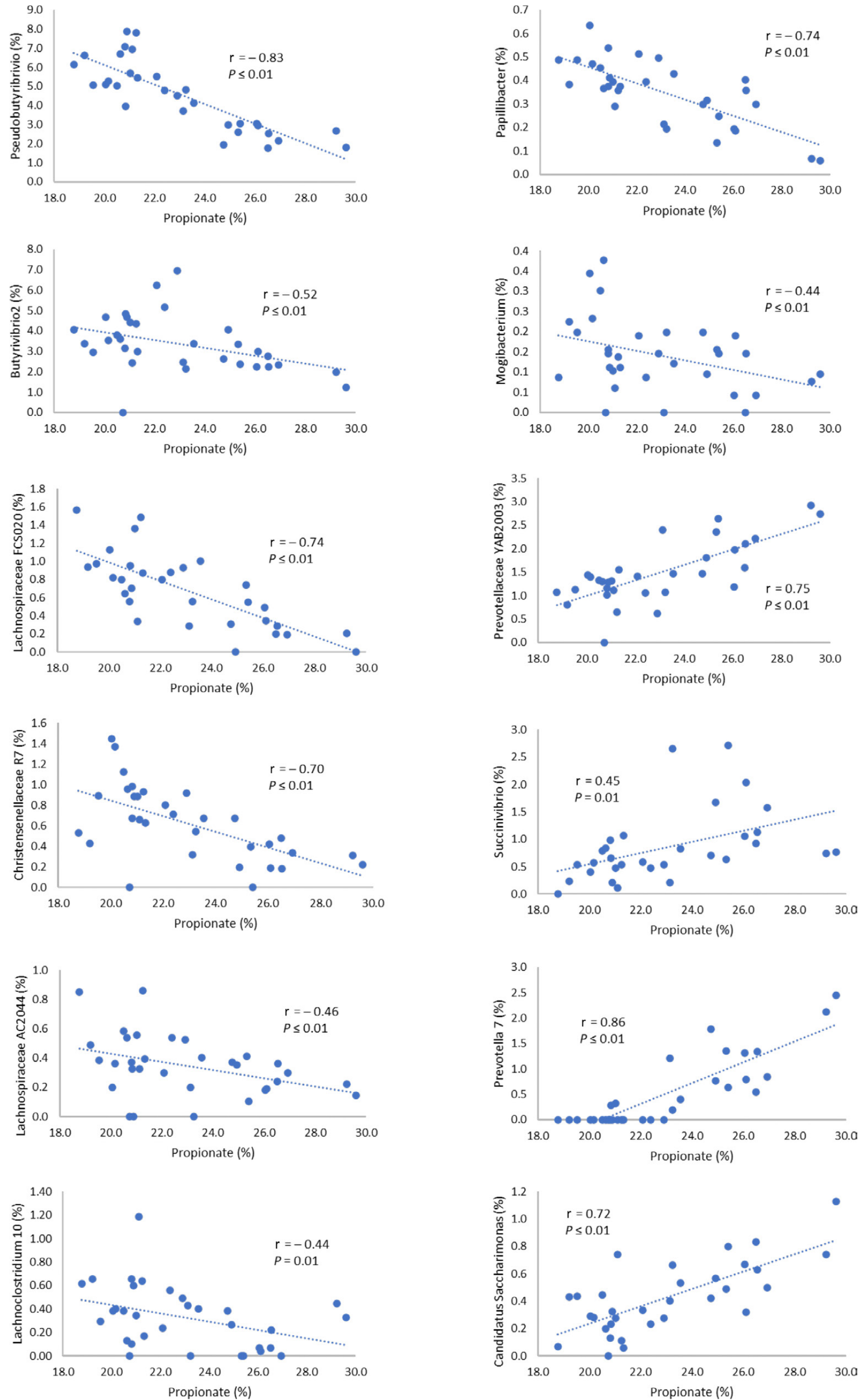

Figure 4. Correlation between molar proportion of propionate in ruminal fluid and relative abundance of genera in solid fraction. Only genera influenced by unprotected choline chloride supplementation (Cho) or its interaction with dietary NDF concentration (NDF × Cho) were analyzed.

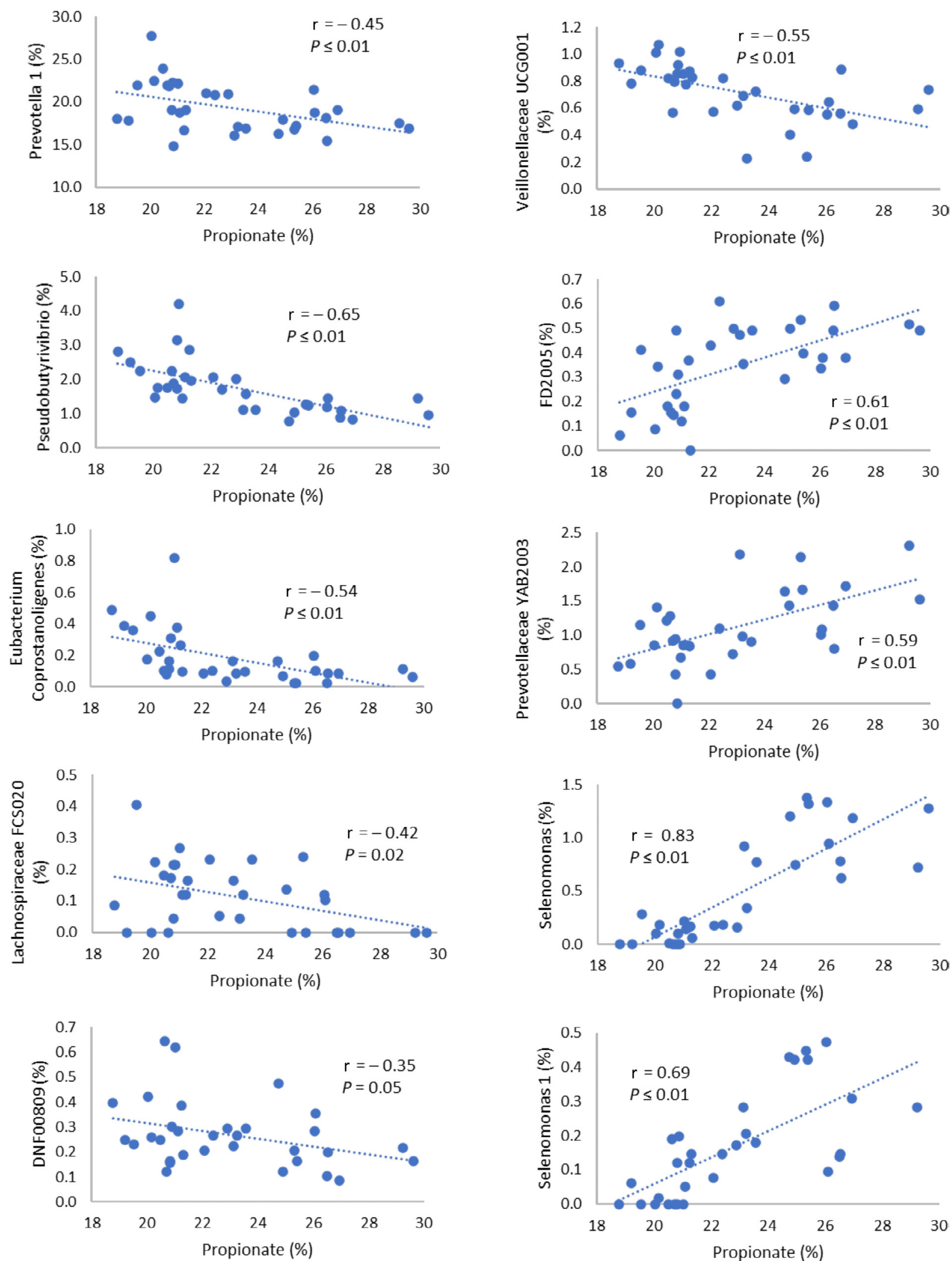

Figure 5. Correlation between molar proportion of propionate in ruminal fluid and relative abundance of genera in liquid fraction. Only genera influenced by unprotected choline chloride supplementation (Cho) or its interaction with dietary NDF concentration (NDF × Cho) were analyzed.

molar proportion and the abundances of orders and genera that were influenced by either Cho or NDF × Cho. In the solid fraction (Figure 3, panel A) we found that order *Clostridiales* was negatively correlated with propionate molar proportion; consistently, genera *Pseudobutyrivibrio*, *Butyrivibrio 2*, *Lachnospiraceae FCS020*, *Christensenellaceae R7*, *Lachnospiraceae AC2044*, *Lachnoclostridium 10*, *Papillibacter*, and *Mogibacterium*, all of them belonging to order *Clostridiales*, were negatively correlated with propionate molar proportion as well (Figure 4). Similarly, in the liquid fraction (Figure 3, panel B) we found a negative correlation between molar proportion of propionate and relative abundance of orders *Fibrobacterales*, *Coriobacteriales*, and *Rickettsiales*; and genera *Prevotella 1*, *Pseudobutyrivibrio*, *Eubacterium coprostanoligenes*, *Lachnospiraceae FCS020*, *DNF00809*, and *Veillonellaceae UCG001* (Figure 5).

Relevance of *Fibrobacterales* in fiber degradation has been shown by its large share of cellulase transcripts in metagenomics evaluations (Söllinger et al., 2018). Moreover, *Clostridiales* are associated with fiber degradation as shown by their greater abundance during the dynamics of colonization of ryegrass over time (Huws et al., 2016) and their involvement as one of the main orders participating in ruminal synthesis of hydrogen (Greening et al., 2019), which is primarily used for synthesis of methane that has been identified as the main hydrogen sink in the rumen at the expenses of other hydrogen consuming pathways such as propionate synthesis (Ungerfeld, 2020). Therefore, the negative correlation of *Fibrobacterales* and *Clostridiales* with propionate synthesis is an expected outcome that is also consistent with the decrease in acetate molar proportion observed as an effect of Cho in our companion study (Arce-Cordero et al., 2021).

On the other hand, a positive correlation with molar proportion of propionate was found for relative abundances of bacteria of the order *Selenomonadales* in both solid and liquid fractions (Figure 3, panels A and B, respectively), and genera *Prevotellaceae YAB2003*, *Succinivibrio*, *Prevotella 7*, and *Candidatus* Saccharimonas in solid fraction (Figure 4) and *FD2005*, *Prevotellaceae YAB2003*, *Selenomonas*, and *Selenomonas 1* in liquid fraction (Figure 5). Given the role of order *Selenomonadales* and particularly bacteria of genera *Selenomonas* in the synthesis of propionate from succinate (Scheifinger and Wolin, 1973), our results indicate that Cho effects on ruminal fermentation may favor this pathway, especially for bacteria present in the liquid fraction.

Moreover, bacteria of other orders such as *Bacteroidales* in the case of *Prevotella* and *Saccharimonadales* in the case of *Candidatus* Saccharimonas, also play roles in fermentation of nonstructural carbohydrates (Purushe et al., 2010; Ogunade et al., 2019; respectively) and may influence part of the response to Cho on ruminal fermentation, particularly in bacteria present in the solid fraction. Interestingly, the most abundant genus in the liquid fraction (*Prevotella 1*) presented a negative correlation with propionate molar proportion, which was the opposite trend to that observed in other phylotypes of *Prevotella* whose abundance was positively associated with propionate in the present study. Similar results were reported by Liu et al. (2017), who found that *Prevotella 1* was associated with lower propionate concentrations and greater synthesis of methane as opposed to other phylotypes of *Prevotella*, suggesting different propionate-producing abilities among phylotypes.

It is not known whether the effects of choline on propionate synthesis may have other implications on ruminal dynamics of hydrogen sinks that were not evaluated in the present study. Therefore, the influence of choline degradation on such reducing pathways would need to be evaluated as a possible mode of action of choline on ruminal fermentation.

CONCLUSIONS

Our results indicate that ruminal fermentation of unprotected choline chloride has an effect on the ruminal microbiome. Most of the effects are observed on taxonomic relative abundance because unprotected choline chloride primarily decreases abundance of bacterial taxa involved in fiber degradation and increases abundance of bacteria mainly involved in degradation of nonstructural carbohydrates and synthesis of propionate, particularly when a diet with 30% NDF is provided in comparison to a 40% NDF diet. Further research would be needed to study the effects of unprotected choline chloride on cows' ruminal metabolism of hydrogen, synthesis of methane, milk production, and milk-fat synthesis.

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