Supply of palmitic, stearic, and oleic acid changes rumen fiber digestibility and microbial composition

Austin Sears,1* Fernanda Hentz,2 Jonas de Souza,3 Benjamin Wenner,4 Robert E. Ward,5 and Fernanda Batistel2*

1Department of Animal Sciences, Utah State University, Logan, UT, 53706
2Department of Animal Sciences, University of Florida, Gainesville, FL, 32611
3Perdue Agribusiness, Salisbury, MD, 21804
4Department of Animal Sciences, The Ohio State University, Columbus, OH, 43210
5Department of Nutrition, Dietetics and Food Sciences, Utah State University, Logan, UT, 43210

ABSTRACT

The concept that fat supplementation impairs total-tract fiber digestibility in ruminants has been widely accepted over the past decades. Nevertheless, the recent interest in the dietary fatty acid profile to dairy cows enlightened the possible beneficial impact of specific fatty acids (e.g., palmitic, stearic, and oleic acids) on total-tract fiber digestibility. Since palmitic, stearic, and oleic acids are the main fatty acids present in ruminal bacterial cells, we hypothesize that the dietary supply of these fatty acids will favor their incorporation into the bacterial cell membranes, which will support the growth and enrichment of fiber-digesting bacteria in the rumen. Our objective in this experiment was to investigate how dietary supply of palmitic, stearic, and oleic acid affect fiber digestion, bacterial membrane fatty acid profile, microbial growth, and composition of the rumen bacterial community. Diets were randomly assigned to 8 single-flow continuous culture fermenters arranged in a replicated 4 × 4 Latin square with four 11-day experimental periods. Treatments were: 1) a control basal diet without supplemental fatty acids (CON); 2) the control diet plus palmitic acid (PA); 3) the control diet plus stearic acid (SA); and 4) the control diet plus oleic acid (OA). All fatty acid treatments were included in the diet at 1.5% of the diet (dry matter [DM] basis). The basal diet contained 50% orchardgrass hay and 50% concentrate (DM basis) and was supplied at a rate of 60 g of DM/day in two equal daily offers (0800 and 1600 h). Data were analyzed using a mixed model considering treatments as fixed effect and period and fermenter as random effects. Our results indicate that PA increased in vitro fiber digestibility by 6 percentage units compared with the CON, whilst SA had no effect and OA decreased fiber digestibility by 8 percentage units. Oleic acid decreased protein expression of the enzymes acetyl CoA carboxylase compared with CON and PA, while fatty acid synthase was reduced by PA, SA, and OA. We observed that PA, but not SA or OA, altered the bacterial community composition by enhancing bacterial groups responsible for fiber digestion. Although the dietary fatty acids did not affect the total lipid content and the phospholipid fraction in the bacterial cell, PA increased the flow of anteiso C13:0 and anteiso C15:0 in the phospholipidic membrane compared to the other treatments. In addition, OA increased the flow of C18:1 cis-9 and decreased C18:2 cis-9, cis-12 in the bacterial phospholipidic membranes compared to the other treatments. Palmitic acid tended to increase bacterial growth compared to other treatments, whereas SA and OA did not affect bacterial growth compared with CON. To our knowledge, this is the first research providing evidence that palmitic acid supports ruminal fiber digestion through shifts in bacterial fatty acid metabolism that result in changes in growth and abundance of fiber-degrading bacteria in the microbial community.

Key words: fatty acids, fibrolytic bacteria, microbial community, phospholipidic membrane

INTRODUCTION

Fiber digestion is a key driver to optimize the efficiency of milk and meat production by ruminants, as well as the profitability and sustainability of the production systems (Adesogan et al., 2019). Improvements in animal performance associated with fiber digestion are due to a positive effect on feed intake and energy absorption. A one-unit increase in fiber digestibility (measured as NDF) was associated with a 0.17 kg increase in DMI and a 0.25 kg increase in 4% FCM in dairy cows (Oba and Allen, 1999). Furthermore, in-
creasing fiber digestibility can support the sustainable production of ruminants by decreasing the intensity of greenhouse gas emissions and nitrogen excretion per unit of food produced, as well as diminishing land and water use, without sacrificing lucrativeness (White, 2016; Hassanat et al., 2017). Although supplementing fat to dairy cows is a common practice to boost dietary energy density and animal performance, it has been extensively acknowledged since the 1950s that supplemental fat impairs fiber digestibility (Palmquist and Jenkins, 2017). However, specific characteristics of individual fatty acids, such as the number of carbons and unsaturation, may differently impact the digestive process. Recent interest in dietary fatty acid profile and its metabolic and physiological effects on dairy cattle suggested that not all long-chain fatty acids negatively impact total-tract fiber digestibility (Weld and Armentano, 2017). Interestingly, feeding specific fatty acids can even enhance total-tract fiber digestibility. For instance, feeding a palmitic acid-enriched supplement (1.5% diet DM) to mid-lactating dairy cows increased NDF digestibility by 4 to 5 percentage units compared with a nonfat-supplemented diet (de Souza and Lock, 2018; Sears et al., 2020). Similarly, studies with early-lactating cows receiving either a palmitic and stearic acid supplement (2% diet DM; 47% stearic and 37% palmitic acid) or a fat blend (1.5% diet DM) containing increasing levels of oleic acid (10 to 30%) replacing palmitic acid (60–80%) reported higher total-tract fiber digestibility compared with a nonfat-supplemented diets (Piantoni et al., 2015; de Souza et al., 2021). What remains unknown is the mechanism underlying how palmitic, stearic, and oleic acid enhance fiber digestion. Since the rumen is the main site of fiber fermentation in ruminants, it is plausible to speculate that the modulatory effects of these fatty acids on fiber digestion are rumen related.

Fiber digestion in the rumen is predominantly done by bacteria (Varga and Kolver, 1997), and alterations in bacterial growth, community composition, and activity can enhance fiber digestion. In non-rumen bacteria, it is well established that exogenous long-chain fatty acids are incorporated into the cell membranes minimizing energy expenditure with fatty acid synthesis (Parsons et al., 2014; Herndon et al., 2020). Nevertheless, to the best of our knowledge, there has been no previous studies examining the incorporation of exogenous fatty acids into the membrane of rumen bacteria. Since palmitic, stearic, and oleic acid represents ~68% of fatty acid composition of mixed rumen bacteria (Or-Rashid et al., 2007), we hypothesize that the exogenous supply of these fatty acids will favor their incorporation into the bacterial cell membranes, which will support growth and enrichment of fiber-digesting bacteria in the rumen. Our objective in this experiment was to investigate how the dietary supply of palmitic, stearic, and oleic acid affect fiber digestion, bacterial membrane fatty acid profile, microbial growth, and composition of the rumen bacterial community.

MATERIALS AND METHODS

Experimental Design and Diets

The Institutional Animal Care and Use Committee at Utah State University, Logan, UT approved the care and handling of ruminal fluid donor animals (Protocol no. 10145). Diets were randomly assigned to 8 single-flow continuous culture fermenters, designed according to Teather and Sauer (1988). The treatments were arranged in a replicate 4 × 4 Latin square design with 4 11-d experimental periods, consisting of 7 d for diet adaptation and 4 d for sample collection. The basal diet contained 50% orchardgrass hay and 50% concentrate (DM basis) and was supplied at a rate of 60 g of DM/day in 2 equal daily offers (0800 and 1600 h). Treatments were: 1) the basal diet without supplemental fatty acids (CON); 2) the basal diet plus palmitic acid (PA; 99% C16:0; Catalog no. P0500, Sigma-Aldrich); 3) the basal diet plus stearic acid (SA: 99% C18:0; Catalog no. S4751, Sigma-Aldrich); and 4) the basal diet plus oleic acid (OA; 99% cis-9 C18:1; Catalog no. O1008, Sigma-Aldrich). All fatty acid treatments were included at 1.5% of the diet (DM basis). The level of palmitic acid inclusion is based on Wenner and St-Pierre (2019); they observed maximum NDF digestibility in vitro with the inclusion of 1–2% of palmitic acid in the diet. Since there is a lack of studies investigating levels of stearic and oleic acid on rumen fermentation and animal performance, we chose the level of palmitic acid and maintained the same feeding level across treatments to avoid a confounding effect of fatty acid dose. Notably, the dose of 1.5% of supplemental fatty acids in the diet falls within the range of fatty acid inclusion used in recent studies (Dos Santos Neto et al., 2021), and this dose is also commonly used in the field. Dietary ingredients and chemical composition are presented in Table 1. The concentrate was grounded to pass a 2 mm screen (Wiley mill; Thomson Scientific, Philadelphia, PA) and the orchardgrass hay was pelleted. Concentrate and hay were weighed into labeled plastic cups, then the fatty acid treatments were added and mixed with the diet. Cups were sealed and stored at 4°C before administration.
Continuous Culture System Operation

At the beginning of each period, ruminal content was collected before morning feeding (0630 h) from 2 rumen-cannulated lactating cows fed a lactating diet (50% forage and 50% concentrate). The ruminal digesta was collected from the ventral, central, and dorsal areas of the rumen and then filtered through double-layered grade 60 cheese cloth into pre-warmed 39°C containers. The containers were kept at 39°C in a pre-heated water bath and immediately transported to the laboratory. The ruminal fluid was homogenized and mixed with artificial saliva (Weller and Pilgrim, 1974) containing 0.4 g/L of urea in a 1:1 proportion and maintained at 39°C. The ruminal fluid and artificial saliva mixture was poured into each fermenter until it cleared the overflow spout. During the whole experiment, fermenters were maintained at 39°C, carbon dioxide (20 mL/min) was continuously infused to maintain anaerobic conditions, and the fermenters content was uninterruptedly stirred by a central paddle set at a speed of 50 rpm. Artificial saliva was continuously bubbled with carbon dioxide to maintain anaerobic condition and was delivered continuously at 10%/hour fractional dilution rate using peristaltic pumps. The pH in the vessels was automatically measured every 10 min and values ranged between 6.00 and 6.70. Clarified ruminal fluid (centrifuged at 15,000 × g, 4°C, 15 min, and autoclaved) was added in a 1:20 dilution of artificial saliva for the first 3 d of each experimental period to better adapt protozoa to fermenters (Wenner et al., 2017). On d 5 of each period, fermenters were dosed with 50 mg of ammonium sulfate enriched with 10% 15N (Catalog no. 348473, Sigma-Aldrich). Additionally, the same ammonium sulfate was added to the artificial saliva at 25 mg/L from d 5 until the end of the experiment for a desired enrichment of 0.2% atom excess. Samples of the outflow effluent were collected before 15N infusion to be used as background for microbial growth calculations.

Sample Collection and Analysis

Diet samples were collected in the last 4 d of each period, composited by period, and dried in a forced-air oven at 55°C for 72 h. On d 8–11 of each period, outflow effluent was collected on ice to prevent further fermentation. Four hundred mL of outflow effluent per fermenter was frozen at −20°C and freeze-dried (FreeZone 12, Labconco). Dried samples of diet and outflow effluent were ground with a Wiley mill (1-mm screen; Arthur H. Thomas) before analyses. Diet and outflow effluent were analyzed for DM (method 934.01; AOAC, 2000), ash (method 942.05; AOAC, 2000), and NDF (Van Soest et al., 1991) with use of heat-stable amylase (Catalog no. FAA, Ankom Technology) and sodium sulfite (Catalog no. S0505, Sigma-Aldrich). The NDF values were corrected for ash. Dietary nitrogen was determined by the Kjeldahl method (method 988.05; AOAC, 2000). Dietary starch was determined according to (Hall, 2009), and the fatty acid content was determined using the one step method of Sukhija.
and Palmquist (1988) with the adaptations proposed by Lock et al. (2013).

Twenty mL of effluent was added to a bottle containing 1 mL of 6 N HCl and then frozen at –20°C. Samples were centrifuged (15,000 × g, 4°C, 15 min) and supernatant was used to quantify short-chain fatty acids (SCFA) using a gas chromatograph (Nexis GC-2030, Shimadzu Corporation) equipped with a capillary column (30 m × 0.53 mm i.d., 0.50 μm phase thickness, Restek). Crotonic acid (Catalog no. 113018, Sigma-Aldrich) diluted in toluene was used as an internal standard and chromatograph conditions were as follows: helium 1.7 mL/min; oven temperature was 110°C held for 2.1 min, which was then increased by 25°C/min to 200°C; flame ionization temperature 220°C; split injection ratio 1/20; injection volume, 1 μL. Peaks were identified by the comparison of retention times with SCFA standards (catalog nos. A6283, I1754, 15374, 240370, 129542 and CRM46975, Sigma-Aldrich; 149300025 and 108110010, Thermo Scientific). In the method used, isovalerate co-elutes with 2-methylbutyrate, and the 2 could not be distinguished in the present study.

Bacterial cells from the effluent (500 mL) were isolated by centrifugation using a procedure adapted from Siddons et al. (1982) and Legay-Carmier and Bauchart (1989). Briefly, samples were kept at 4°C overnight to allow the detachment of bacteria from the feed particles and then centrifugated at 3500 × g for 5 min at 4°C to remove eukaryotes and feed particles. Subsequently, the supernatant was centrifugated at 20000 × g for 30 min at 4°C and resuspended once with NaCl solution (0.9%) containing Tween 80 (1g/L; catalog no. BP338–500, Fisher Scientific) and twice with distilled water. An aliquot of the bacterial cell was reserved for N analysis and the remaining cells were freeze-dried and 500 mg were used to extract the lipids using the method described by Folch et al. (1957) with adaptations. Briefly, lipids were extracted using methanol, chloroform, and a 2% NaCl solution. The proportion methanol:chloroform:NaCl was 1:2:1. Samples were dried under nitrogen and weighed to obtain total lipid amount, then reconstituted in 0.5 mL hexane:methyl tert-butyl ether:acetic acid 100:3:0.3. Lipid classes were separated by a solid-phase extraction method using a vacuum manifold kit (Catalog no. RE28298-VM, Restek) and aminopropyl SPE columns (Catalog no. 60108–432, Thermo Scientific) according to the procedures of Agren et al. (1992). After separation, samples were dried under nitrogen and weighed to obtain the phospholipidic fraction. Fatty acid profile of the phospholipidic fraction were determined using the 2 step method of Sukhija and Palmquist (1988) with the adaptations proposed by Lock et al. (2013). The fatty acid were prepared by adding 5% methanolic sulfuric acid to the samples. The FAME was filtered through anhydrous sodium sulfate, solvents were removed under nitrogen flux at 37°C, the FAME weighed, and a 1% solution of n-hexane prepared on a weight basis. C10-10 C17:1 (Catalog no. H8896, Sigma-Aldrich) diluted in toluene was used as an internal standard. The FAME were determined by gas chromatograph (Nexis GC-2010, Shimadzu). Fatty acids were separated using a CP Sil 88 column (100 m × 0.25 mm, 0.2 μm of film thickness, Agilent Technologies). Hydrogen was used as the carrier gas at a constant rate of 1 mL/min. The temperature of the gas chromatograph oven was maintained at 45°C for 4 min, increased at 13°C/min to 175°C and held for 27 min, and increased at the rate of 4°C/min to a final temperature of 215°C and held for 35 min. Peaks were identified by the comparison of retention times with fatty acid methyl ester standards (catalog nos. GLC-463 and UC-59-M, Nu-Check-Prep Inc.; and 47080-U, Sigma-Aldrich). The fatty acid flow was calculated based on the procedures described by Glasser et al. (2007), converting the fatty acid weight to a molar basis and accounting for glycerol and phosphate contributions to phospholipids.

Ammonia N in effluent samples was determined by colorimetric analysis (Chaney and Marbach, 1962). Bacterial cells and effluent were analyzed for total N and 15N. Dried effluent samples (50 mg) were weighed, wetted with distilled water, adjusted with 10 N NaOH to a pH > 10, and dried at 90°C for 16 h to remove ammonia N (Hristov et al., 2001). Bacterial and effluent samples were analyzed for 15N by the stable isotope laboratory (Utah State University, Logan) according to procedures described by Noftsger et al. (2003). The 15N background was subtracted from the 15N enrichment after 15N infusion to determine the atom percentage excess of 15N. The ammonia flow (g/day) was calculated as the concentration of ammonia N × total effluent flow. The NAN flow (g/day) was calculated as total N - ammonia flow. Bacterial N flow (g/day) was calculated as (NAN flow × 15N atom percentage excess of effluent NAN)/(15N atom percentage excess of bacteria). Bacterial nitrogen per NDF digestibility was calculated as bacterial N flow/NDF degraded. The RUP was calculated as non-ammonia non-bacterial N/NAN and RDP = 100 - RUP.

Bacteria cells were used to determine the protein expression of acetyl CoA carboxylase (ACC, Enzyme commission [EC] number 6.4.1.2) and fatty acid synthase (FAS, EC number 2.3.1.85), which are key regulatory enzymes in the de novo fatty acid synthesis pathway. Protein was obtained by homogenizing 50 mg of bacterial cells with 0.4 g of sterile glass beads and a lysis buffer (Snelling and Wallace, 2017) using a bead
beater (Bead Ruptor 4, Omni International). Extracted protein concentrations were determined using the Qubit Fluorometer (Invitrogen). Protein expression was determined using the WES technology (ProteinSimple) following the manufacturer’s recommendations for a 25-well plate protocol using a 66–440 kDa kit. Briefly, 0.5 mg/mL of protein was used, and target proteins were immune-probed with primary antibodies for ACC (Catalog no. 3676S, Cell Signaling Technologies), FAS (Catalog no. 3189S, Cell Signaling Technologies), and β-actin (catalog no. 115777, Abcam), followed by HRP-conjugated secondary antibodies provided in the WES kit. The primary antibodies were diluted using an antibody diluent (Protein Simple) at a 1:50, 1:10, and 1:25 ratio, respectively for ACC, FAS, and β-actin. Digital image was analyzed with Compass software (Protein Simple), and data was normalized to β-actin.

Total genomic DNA was extracted from outflow samples using the bead beating plus column method (Yu and Morrison, 2004), and DNA was quantified using a Qubit Fluorometer. PCR was performed using universal primers flanking the variable 4 (V4) region of the 16S rRNA (Kozich et al., 2013). Samples were quantified with a Qubit fluorometer, pooled on an equimolar basis, and sequenced with MiSeq v3 kit (2 × 300 cycles, Illumina) according to the manufacturer’s protocol. All sequences were demultiplexed on the Illumina MiSeq system. Further, sequence processing was performed using mothur v1.45.1 (Schloss et al., 2009) following the protocol described by Kozich et al. (2013). Briefly, paired-end sequences were combined into contigs, and poor-quality sequences were removed. Bacterial sequences were aligned and classified using the SILVA 16S rRNA database (Pruesse et al., 2007). All sequences were grouped into 97% OTUs by uncorrected pairwise distances and furthest neighbor clustering. Bacterial communities were normalized to equal sequence counts near the lowest sample, and these normalized OTU tables were used in all further analyses.

**Statistical Analysis**

Parts of the microbiota statistical analyses were carried out in R (vegan package). Total microbial community structure (Bray-Curtis) and composition (Jaccard) were calculated from normalized OTU data and visualized by non-metric multidimensional scaling (NMDS) plots. The PERMANOVA was run to determine the differences in community structure and composition between treatments by using the adonis function in vegan, with the Benjamini-Hochberg correction for multiple comparisons.

Data for digestibility, rumen nitrogen, fatty acids, α diversity, and relative abundance were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute, Inc. Cary, NC) according to the following model:

\[ Y_{ijk} = \mu + p_i + f_j + T_k + e_{ijk}, \]

where \( Y_{ijk} \) = variable of interest, \( \mu \) = overall mean, \( p_i \) = random effect of period (\( i = 1 \) to 4), \( f_j \) = random effect of fermenter (\( j = 1 \) to 8), \( T_k \) = fixed effect of treatment (\( k \) = Control, Palmitic, Stearic, and Oleic), \( e_{ijk} \) = residual error. The normality of the residuals was checked with normal probability and box plots and homogeneity of variances with plots of residuals vs. predicted values. A protected least significant difference was used for mean separation. Significance was declared at \( P \leq 0.05 \) and tendency at \( P \leq 0.10 \).

**RESULTS**

**In vitro NDF digestibility, SCFA, and nitrogen flow**

Digestibility of NDF, total SCFA flow, and valerate yield increased with PA and decreased with OA compared with CON and SA (\( P < 0.01 \); Figure 1A, 1B and 1F). Compared with CON and PA, SA and OA decreased propionate flow (\( P = 0.03 \); Figure 1D). Isobutyrate flow decreased with SA and OA compared with CON and PA (\( P < 0.01 \); Figure 1G). The flow of acetate, butyrate and isovalerate were not affected by treatments (Figure 1C, 1E and 1H).

The concentration of ammonia nitrogen in the effluent was not affected by treatments (Table 2). Bacterial nitrogen in the outflow effluent tended to be greater with PA relative to other treatments (\( P = 0.09 \)), while flow of bacterial nitrogen per unit of NDF digestibility was not affected by the treatments. Total nitrogen flow, and non-ammonia nitrogen and non-ammonia nonbacterial nitrogen in the effluent nitrogen flow were not different between treatments. Palmitic acid increased RDP and decreased RUP compared with other treatments (\( P = 0.05 \)).

**Bacterial ACC and FAS protein expression, bacterial phospholipid fatty acid**

Bacterial protein expression of ACC was reduced by OA compared with CON and PA (\( P < 0.01 \); Figure 2A). Protein expression of FAS was reduced by all fatty acid treatments compared with CON (\( P < 0.01 \); Figure 2B). More specifically, PA reduced FAS expression compared with SA, and OA reduced FAS expression compared with PA and SA.

The bacterial phospholipid fatty acid flow is presented in Table 3. The fatty acid treatments did not alter total bacterial phospholipid content compared
with CON (Table 3). Regarding individual fatty acids, PA increased anteiso-C13:0 ($P = 0.01$), anteiso-C15:0 ($P = 0.05$) and tended to increase C17:0 ($P = 0.07$) compared with the other treatments. OA increased C18:1 cis-9 content compared with CON and SA ($P = 0.02$). In contrast, OA decreased C18:2 cis-9, cis-12 compared with the other treatments ($P = 0.05$) and tended to decrease C18:3 cis-9, cis-12, cis-15 compared with CON and PA ($P = 0.09$).

### 16S rRNA data acquisition and analysis

The sequencing of the bacterial 16S rRNA of the effluent samples generated an average of 46,996 high quality sequences per sample (Supplementary Table S1). Sequence coverage met a Good’s coverage greater than 99.5% for all samples, implying that sampling provided sufficient OTU coverage to accurately describe the bacterial composition in each treatment.

### Richness, Diversity and Composition of the bacterial communities

The richness indices Chao and Ace were not affected by treatments (Figure 3A and 3B). Diversity of the microbial community increased with PA supplementation relative to the CON, SA, and OA treatments based on the Shannon index ($P = 0.05$; Figure 3C). Similarly, we observed that PA tended to increase the Inverse Simpson index ($P = 0.08$; Figure 3D) compared with CON and SA. For β-diversity analysis, we did not observe a treatment effect on Bray-Curtis and Jaccard distances in the PERMANOVA analysis (Supplementary Table S2). The PCOA and NMDS plots based on the Bray-Curtis similarity showed overlapping of points (Supplementary Figure S1 and S2), indicating that the β-diversity composition of the bacterial community was not significantly affected by treatments.

At the phylum level, 6 bacterial phyla were identified in the effluent samples (Figure 4). Regardless of dietary treatment, the bacterial community composition was dominated by the phylum Firmicutes (48.2%) and Bacteroidetes (34.8%). Bacteroidetes were increased by PA compared with the other treatments ($P = 0.04$). PA increased Fibrobacteres when compared with OA ($P = 0.01$). The abundance of the phylum Actinobacteria, Desulfovibrio, Firmicutes, Spirochaeta, and Verrucomicrobiota were not affected by treatments.

Twenty-seven bacterial families represented 95% of the abundance at family level (Table 4). Prevotellaceae and Lachnospiraceae had the largest relative abundance across all treatments accounting for 25.2% and 29.9% of total sequences, respectively. PA increased the relative abundance of Prevotellaceae compared with the other treatments ($P = 0.03$). OA decreased the relative
abundance of *Fibrobacteraceae* in comparison with PA ($P = 0.01$). Dietary PA tended to decrease the relative abundance of *Bacillaceae* ($P = 0.10$) compared with CON. The FA treatments had no effect on the relative abundance of the other families identified from the 16S gene sequencing.

At the genus level, *Prevotella*, *Butyrivibrio*, and *Ruminococcus* were the most abundant genera, representing 19.1, 5.0, and 4.3% of the total sequences, respectively. Dietary PA supplementation increased the relative abundance of *Prevotella* compared with other treatments ($P < 0.01$; Figure 5A). The relative abundance of the *Fibrobacter* genus was higher in the PA compared with OA ($P = 0.01$; Figure 5B), but not different from CON and SA, which were intermediate. OA increased the relative abundance of *Megasphaera*, *Anaerovibrio*, *Lachnobacterium*, and *Pseudobutyrivibrio* ($P \leq 0.05$; Figure 5C-F). OA tended to increase relative abundance of *Desulfovibrio* compared with PA and SA ($P = 0.08$; Figure 5G). SA increased the relative abundance of *Acidaminococcus* compared with OA ($P = 0.02$; Figure 5H). The relative abundance of the other bacterial genera was not affected by the dietary treatments.

**DISCUSSION**

Enhancing fiber digestibility by ruminant animals is the research focus of several disciplines because it augments the efficiency of food production and sustainability of the production systems. The recent interest in dietary fatty acid profile to dairy cows enlightened the possible advantageous impact of specific fatty acids (e.g., palmitic, stearic, and oleic acid) on total-tract fiber digestibility (Piantoni et al., 2015; de Souza et al., 2018; Sears et al., 2020; de Souza et al., 2021). Because fiber is primarily digested in the rumen, here we used continuous culture fermenters, which mimic rumen fermentation, to explore the hypothesis that dietary supply of palmitic, stearic, and oleic acid enhances fiber digestion. We postulate that these fatty acids are incorporated into bacterial cell membranes and consequently support bacterial growth and enrichment of fiber-digesting bacteria.

Our results show that palmitic acid increased fiber digestibility by 6 percentage units compared with the control, while stearic did not affect and oleic acid decreased fiber digestibility by 8 percentage units. A previous meta-analysis reported an increase of 4.5 percentage units in total-tract fiber digestibility of dairy cows when the inclusion of a palmitic acid-enriched supplement (>80% palmitic acid) ranged from 0.75 to 3.0% of diet DM (mean of 1.81%) (Dos Santos Neto et al., 2021). However, the positive effect of palmitic acid on fiber digestibility is not entirely consistent in the literature. For example, Rico et al. (2014) did not observe any effect on fiber digestibility when 2% of a palmitic acid-enriched supplement (85% palmitic acid) was added to the diet of dairy cows. The inconsistencies may be attributed to the total fatty acid concentration in the diet as well as source and level of fiber in the diet. Compared with palmitic acid, fewer studies investigated the inclusion of stearic and oleic acid-enriched supplements into the diet of dairy cows. The lack of response to stearic acid in our study is consistent with the observations of Boerman et al. (2017), which observed no effect on total-tract fiber digestibility when cows were supplemented with a stearic-acid enriched supplement (93% stearic acid) at 0, 0.80, 1.50, or 2.30% of diet DM. In contrast, Piantoni et al. (2015)
reported that the inclusion of 2% diet DM of a stearic acid-enriched supplement (98% stearic acid) tended to increase total-tract fiber digestibility compared with a diet without supplemental fatty acids. de Souza et al. (2021) observed that fat blends (fed at 1.5% diet DM) containing increasing levels of oleic acid (10 to 30%) replacing palmitic acid (60–80%) increased total-tract fiber digestibility compared with a nonfat-supplement diet. To note, Boerman et al. (2017) and Souza et al. (2021) reported a linear increase in cows’ feed intake in response to the increasing levels of stearic and oleic acid, respectively; the higher feed intake may have affected the digestibility by increasing the passage rate (NASEM, 2021). In general, we have chosen a 10% passage rate in our in vitro system to simulate intakes and passage of high-producing cows. The passage rate of up to 10%/h is higher than most in vivo studies, but the high NDF degradation may suggest that some particulates are settling and passing slower in the system. Our findings indicate that when palmitic, stearic, or oleic were supplied at 1.5% diet DM, only palmitic acid positively affected fiber digestion in the vessels.

It is presumed that the de novo fatty acid synthesis pathway is conserved between eukaryotes and bacteria (Cronan and Thomas, 2009). Two key enzymes are involved in the de novo lipogenesis, ACC and FAS. The multisubunit ACC catalyzes the first step in the fatty acid synthesis, and its reaction can be divided into 2 partial reactions (Cronan and Waldrop, 2002). In the first step, biotin is carboxylated via ATP consumption and in the second step, the carboxyl group is transferred to acetyl-CoA, yielding malonyl-CoA. Subsequently, FAS converts malonyl-CoA to long-chain fatty acids by a repeated cycle of reactions involving condensation, reduction, dehydration, and subsequent reduction of carbon-carbon bonds (Schweizer and Hofmann, 2004). The lower protein expression of ACC following OA supply and of FAS with PA, SA, and OA in our study suggests downregulation of the de novo pathway. In ruminal bacteria, there is a lack of research on the regulation of the fatty acid synthesis pathway by specific fatty acids. In our study, OA decreased the initiation of fatty acid synthesis, whereas the supply of all exogenous fatty acids decreased fatty acid elongation. The ACC regulation is complex in bacteria, and endogenous synthesis varies by bacterial taxa and how exogenous fatty acids regulate endogenous synthesis (Yao and Rock, 2017). Exogenous fatty acids strongly suppress ACC activity and malonyl-CoA levels in Staphylococcus pneumoniae but not in Staphylococcus aureus, showing a regulatory feedback system taxa-specific (Parsons et al., 2011). In a previous study, OA reduced ACC activity in Lactobacillus plantarum compared with PA, SA, and a control growth medium, suggesting a fatty acid-specific effect on the activity of this enzyme (Birnbaum, 1970). Bacteria have evolved several mechanisms to control the formation of new fatty acids and modify the structure of existing fatty acids to minimize energy expenditure and optimize growth (Zhang and Rock, 2008). Controlling the elongation (chain length) of synthesized fatty acids is a point of control of membrane fluidity since increasing long-chain fatty acids decreases membrane fluidity (Russell, 1984). Therefore, we postulate that if bacteria incorporate exogenous fatty acids, a decrease in fatty acid elongation may occur. The reason why PA, SA and OA were able to inhibit the expression of the

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**Figure 2.** Effect of palmitic (PA), stearic (SA), and oleic acid (OA) on protein expression (arbitrary units; AU) of bacterial acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The control (CON) was a basal diet composed of 50% orchardgrass hay and 50% concentrate (DM basis) without supplemental fatty acids. Fatty acid treatments were added to the basal diet at a concentration of 1.5% (DM basis). For treatment effect, means without a common letter differ ($P < 0.05$).
bacterial FAS enzyme while ACC was inhibited only by OA is not completely clear and should be the object of future investigations. Since fatty acids in bacterial cell membranes can originate from the de novo fatty acid synthesis pathway or incorporation of exogenous fatty acids present in the environment (Erwin, 1973), our data suggest that the supply of dietary fatty acids may lead to greater incorporation of exogenous fatty acids into the bacterial cell membranes to maintain membrane homeostasis. Our results also showed that OA decreased both ACC and FAS expression to a greater extent than PA and SA, suggesting a fatty acid-specific effect on the protein expression of these enzymes.

Although the supply of exogenous fatty acids did not affect the total lipid content and the phospholipid fraction in the bacterial pellet, some differences in the phospholipid fatty acid composition were observed. Previously, Jenkins (1993) reported that the total lipid content in the rumen bacterial mass ranges from 10 to 15% of bacterial DM and our observations for all the treatments concur with that range. Bacterial membranes primarily consist of phospholipids that contain a hydrophilic phosphate head group and a hydrophobic tail consisting of 2 fatty acids (Gullett and Rock, 2021). The phospholipidic fraction observed in our study is lower (average of 28% vs. 39% of total bacterial lipid fraction) than previously observed (Garton and Oxford, 1955) and we believe these differences are due to the method used for phospholipid extraction. The high consistency in the phospholipid fatty acid profile across treatments was expected since major modifications in the membrane fatty acid profile occurs in response to key environmental stressors such as acidic conditions (Zhang and Rock, 2008), and no pH differences across treatments were observed in our study (data not shown). Regulating membrane fatty acid composition is essential to maintain membrane fluidity and integrity for normal and efficient microbial functions (Gullett and Rock, 2021). We observed a higher flow of odd and anteiso fatty acids in the palmitic acid treatment.
ear odd-chain fatty acids are formed when propionyl-CoA, instead of acetyl-CoA, is used as primer, while iso and anteiso fatty acids are originated from branch-chain SCFA (Kaneda, 1991). However, in our study, palmitic acid did not influence the flow of propionate and branch-chain SCFA compared with control. Specific groups of bacteria have characteristic composition of odd- and branched-chain fatty acids in their phospholipid fraction. Therefore, variations in the profile of these fatty acids in mixed rumen bacteria mainly reflect changes in the abundance of specific bacterial populations in the rumen (Vlaeminck et al., 2006). Cellulolytic bacteria contain high amounts of iso-fatty acids while higher proportions of anteiso and linear odd-chain fatty acids indicate the presence of bacteria specialized in the fermentation of pectin and sugars (Vlaeminck et al., 2006, Bessa et al., 2009). Palmitic acid increased the proportion of anteiso-C13:0 and anteiso-C15:0 in the bacterial phospholipid fraction, consistent with the increased relative abundance of the Prevotella genus in this treatment. Palmitic acid also increased the proportion of C17:0 in bacterial phospholipid fraction, which is positively correlated with Fibrobacter (Zhang et al., 2017). Therefore, in our study, the changes in bacterial fatty acid flow are more likely associated with changes in the microbial community than SCFA availability. Our observations regarding bacterial lipid content, membrane phospholipid fraction, and fatty acid profile reinforce the idea that bacterial cells tightly regulate the lipids present in the cell membranes.

We observed that palmitic acid, but not stearic acid or oleic acid, enriched bacterial groups responsible for fiber digestion in the microbial community. The relative abundance of the Fibrobacter genus was increased by 1.5- and 3.8-fold with palmitic acid supply compared with the control and oleic acid, respectively, and the Prevotella increased by 1.2-fold relative to the control and oleic acid. These results strengthen the concept that modulation of fiber digestion is fatty acid-specific and mediated, at least partly, by modifications in the structure of the bacterial community involved with fiber breakdown in the rumen. Fibrobacteraceae and Prevotellaceae represented 1.1% and 28%, respectively, of all bacterial sequences identified in the 16S rRNA gene sequencing with palmitic acid supply, which is greater than previously reported in studies that used a forage: concentrate ratio similar to what was used in this study (Xue et al., 2018). Fibrobacter succinogenes is a major cellulose degrader (Krause et al., 2003), and Prevotella spp. is the most abundant bacteria genus in the rumen (Wallace et al., 2019) and substantially contributes to hemicellulose utilization (Emerson and Weimer, 2017). The high relative abundance of Prevotella might be responsible for the greater total production of SCFA, driven by acetate and butyrate levels, in response to the palmitic acid supply in our study. Previous studies observed that both acetate and butyrate are positively correlated with the relative abundance of Prevotella (Watabe et al., 2018; Xue et al., 2018). Furthermore, the genus Prevotella is known for producing key enzymes in the acetate (Deusch et al., 2017) and butyrate synthesis pathways (Wang et al., 2020).

Supplementing oleic acid showed a negative effect on fiber digestibility and microbial community structure by reducing the abundance of the Fibrobacteres at the phylum, family, and genera level. Oleic acid is reported to be more inhibitory than palmitic and stearic acids on the growth of most fibrolytic bacteria possibly due to its unsaturated nature (Enjalbert et al., 2017). Unsaturated fatty acids are recognized to possess antimicrobial activity by increasing membrane permeability and cell lysis, disrupting the electron transport chain and uncoupling oxidative phosphorylation, and

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effect of palmitic (PA), stearic (SA), and oleic acid (OA) on bacterial α diversity index for richness (Chao and Ace) and diversity (Shannon and Inverse Simpson) in response to supplemental palmitic (PA), stearic (SA), and oleic (OA) acid in continuous culture fermenters. The control (CON) was a basal diet composed of 50% orchardgrass hay and 50% concentrate (DM basis) without supplemental fatty acids. Fatty acid treatments were added to the basal diet at a concentration of 1.5% (DM basis). For treatment effect, means without a common letter differ ($P < 0.05$).
Figure 4. Effect of palmitic (PA), stearic (SA), and oleic acid (OA) on relative abundance of bacterial phylum in continuous culture fermenters. The control (CON) was a basal diet composed of 50% orchardgrass hay and 50% concentrate (DM basis) without supplemental fatty acids. Fatty acid treatments were added to the basal diet at a concentration of 1.5% (DM basis). For treatment effect, means without a common letter differ ($P < 0.05$).

Table 4. Effect of palmitic, stearic, and oleic acid supplementation on the relative abundance of ruminal bacterial families in effluent digesta

<table>
<thead>
<tr>
<th>Family</th>
<th>Control</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>SEM</th>
<th>P-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acholeplasmataceae</em></td>
<td>0.43</td>
<td>0.33</td>
<td>0.43</td>
<td>0.34</td>
<td>0.14</td>
<td>0.75</td>
</tr>
<tr>
<td><em>Acidaminococcaceae</em></td>
<td>1.39</td>
<td>1.31</td>
<td>1.32</td>
<td>1.39</td>
<td>0.35</td>
<td>0.12</td>
</tr>
<tr>
<td><em>Anaerovoracaceae</em></td>
<td>1.75</td>
<td>2.10</td>
<td>2.06</td>
<td>2.40</td>
<td>0.34</td>
<td>0.29</td>
</tr>
<tr>
<td><em>Atopobiaceae</em></td>
<td>2.53</td>
<td>2.74</td>
<td>3.15</td>
<td>2.70</td>
<td>0.59</td>
<td>0.59</td>
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<tr>
<td><em>Bacillaceae</em></td>
<td>1.56</td>
<td>1.31</td>
<td>1.51</td>
<td>1.44</td>
<td>0.22</td>
<td>0.23</td>
</tr>
<tr>
<td><em>Bacteroidaceae</em></td>
<td>1.32</td>
<td>1.32</td>
<td>1.12</td>
<td>1.45</td>
<td>0.56</td>
<td>0.20</td>
</tr>
<tr>
<td><em>Christensenellaceae</em></td>
<td>1.88</td>
<td>1.78</td>
<td>1.28</td>
<td>1.45</td>
<td>0.54</td>
<td>0.20</td>
</tr>
<tr>
<td><em>Clostridaceae</em></td>
<td>0.32</td>
<td>0.37</td>
<td>0.27</td>
<td>0.36</td>
<td>0.18</td>
<td>0.11</td>
</tr>
<tr>
<td><em>Desulfovibrionaceae</em></td>
<td>2.70</td>
<td>2.46</td>
<td>3.12</td>
<td>3.74</td>
<td>0.85</td>
<td>0.19</td>
</tr>
<tr>
<td><em>Erysipelotrichaceae</em></td>
<td>2.31</td>
<td>1.68</td>
<td>1.44</td>
<td>1.53</td>
<td>0.36</td>
<td>0.23</td>
</tr>
<tr>
<td><em>F082</em></td>
<td>1.81</td>
<td>1.65</td>
<td>1.41</td>
<td>1.54</td>
<td>0.4</td>
<td>0.79</td>
</tr>
<tr>
<td><em>Fibrobacteraceae</em></td>
<td>0.72</td>
<td>1.08</td>
<td>0.73</td>
<td>0.28</td>
<td>0.25</td>
<td>0.01</td>
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<tr>
<td><em>Hungateiclostridiaceae</em></td>
<td>0.61</td>
<td>0.58</td>
<td>0.65</td>
<td>0.63</td>
<td>0.13</td>
<td>0.94</td>
</tr>
<tr>
<td><em>Lachnospiraceae</em></td>
<td>20.8</td>
<td>19.3</td>
<td>21.4</td>
<td>22.1</td>
<td>1.93</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Lactobacillaceae</em></td>
<td>3.44</td>
<td>2.67</td>
<td>3.55</td>
<td>3.16</td>
<td>0.52</td>
<td>0.55</td>
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<tr>
<td><em>Muribacillaceae</em></td>
<td>1.64</td>
<td>1.43</td>
<td>1.96</td>
<td>2.41</td>
<td>0.29</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Oscillospiraceae</em></td>
<td>1.75</td>
<td>2.17</td>
<td>1.43</td>
<td>1.59</td>
<td>0.4</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Planococccaceae</em></td>
<td>0.91</td>
<td>1.18</td>
<td>1.44</td>
<td>0.89</td>
<td>0.43</td>
<td>0.70</td>
</tr>
<tr>
<td><em>Prevotellaceae</em></td>
<td>24.6</td>
<td>28.0</td>
<td>24.6</td>
<td>24.6</td>
<td>1.61</td>
<td>0.03</td>
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<tr>
<td><em>Rikenellaceae</em></td>
<td>3.71</td>
<td>3.35</td>
<td>2.79</td>
<td>3.06</td>
<td>0.66</td>
<td>0.54</td>
</tr>
<tr>
<td><em>Ruminococcaceae</em></td>
<td>4.94</td>
<td>4.95</td>
<td>5.07</td>
<td>5.23</td>
<td>0.39</td>
<td>0.75</td>
</tr>
<tr>
<td><em>Selenomonadaceae</em></td>
<td>1.50</td>
<td>1.13</td>
<td>1.46</td>
<td>1.59</td>
<td>0.28</td>
<td>0.21</td>
</tr>
<tr>
<td><em>Sporoactinomycetaceae</em></td>
<td>4.06</td>
<td>3.31</td>
<td>3.88</td>
<td>3.18</td>
<td>0.94</td>
<td>0.70</td>
</tr>
<tr>
<td><em>Veillonellaceae</em></td>
<td>4.10</td>
<td>3.91</td>
<td>5.19</td>
<td>4.68</td>
<td>1.01</td>
<td>0.49</td>
</tr>
<tr>
<td><em>WCHB1-41_fa</em></td>
<td>0.82</td>
<td>0.84</td>
<td>0.59</td>
<td>0.82</td>
<td>0.22</td>
<td>0.28</td>
</tr>
</tbody>
</table>

$^1$The control was a basal diet composed of 50% orchardgrass hay and 50% concentrate (DM basis) without supplemental fatty acids. Fatty acid treatments (palmitic, stearic, and oleic acid) were added to the basal diet at a concentration of 1.5% (DM basis).

$^2$P-values refer to the ANOVA results for the main effect of fatty acid treatment.

$^a$ For treatment effect, means without a common letter within the same row differ ($P < 0.05$). Separation only conducted if treatment effect was $P < 0.10$. 
inhibiting membrane enzymatic activities and nutrient uptake (Yoon et al., 2018). However, the absence of a detrimental effect of oleic acid on other fibrolytic bacteria with exception of Fibrobacters in our study is not clear. In several experiments, species belonging to the Fibrobacter, Ruminococcus, and Butyriovibrio genera were negatively affected by dietary fat addition in most cases. However, most of these studies involved supplementation of oils that have in their composition different concentrations of fatty acids, making it difficult to illustrate the effects of individual fatty acids on the rumen microbial community (Zened et al., 2013; Li et al., 2015). Also, some of these studies included fat in the diet at much higher levels than those used in the present study. In our study, all fatty acid treatments were supplemented at the same feeding level, while in most feeding conditions their proportion is not the same. Supplementing oleic acid to dairy cows has been shown to improve fatty acid digestibility and performance (de Souza et al., 2019; Prom et al., 2021), but the dose provided was smaller than used in our study. Further elucidation of the effects of oleic acid on the microbial community composition would benefit from a dose-response study.

Our results indicate that palmitic acid tended to increase bacterial growth compared with other treatments, while stearic and oleic acid did not affect bacterial growth compared with control. Incorporation of preformed fatty acids spares a considerable amount of energy in bacterial fatty acid metabolism; thus, we hypothesized that the spared energy could be used by bacteria to favor its growth. In this study, mixed rumen bacteria were used for the fatty acid profile analysis of the bacterial phospholipid fraction; therefore, identification of specific microorganisms that incorporated dietary palmitic, stearic, and oleic acid into their cellular membrane is not possible. However, because there is a flux of both liquids and solids through the rumen (Van Soest, 1994), microbes must actively multiply to counteract washout to be able to maintain their populations (Henderson et al., 2015). Therefore, bacteria that increased their relative abundance in the bacterial community with the fatty acid treatments are likely those which used the spared energy from exogenous fatty acid incorporation for growth. Fibrobacteraceae and Prevotellaceae had their relative abundance increased with palmitic acid, and Megasphera, Pseudobutyrivibrio, Lachnobacterium and Anaerovibrio with oleic acid, indicating that palmitic and oleic acid slightly changed the structure of the bacterial community through alterations in evenness of these species. However, bacterial nitrogen pool in the effluent, measured as bacterial nitrogen flow, and RDP increased only with palmitic acid supply highlighting the differential role of individual

**Figure 5.** Effect of palmitic (PA), stearic (SA), and oleic acid (OA) on relative abundance of bacterial genera in continuous culture fermenters. The control (CON) was a basal diet composed of 50% orchardgrass hay and 50% concentrate (DM basis) without supplemental fatty acids. Fatty acid treatments were added to the basal diet at a concentration of 1.5% (DM basis). For treatment effect, means without a common letter differ ($P < 0.05$).
fatty acids on bacteria in the rumen. Although oleic acid enriched the abovementioned genera in the bacterial community, these alterations occurred with a concurrent reduction in prevalence of other bacteria, not translating into an increased bacterial nitrogen pool.

CONCLUSIONS

To our knowledge, this is the first research providing evidence that specific dietary fatty acids support fiber digestion in the rumen by shifting the prevalence of fiber digesters in the bacterial community. For conditions where a readily available supply of preformed palmitic acid exists, particularly bacteria involved in fiber breakdown may incorporate these as substrates into their cell membrane lipids. The enrichment of the genera Prevotella and Fibrobacter in response to palmitic acid supply suggests that this fatty acid enabled these microorganisms to effectively compete and outgrow other genera in the continuous culture system. Further elucidation of the role of palmitic, as well as other fatty acids, on rumen bacterial membrane fatty acid metabolism and growth would benefit from studies using pure culture bacteria and carbon-labeled fatty acids.

ACKNOWLEDGMENTS

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REFERENCES


ORCIDS

Austin Sears @ https://orcid.org/0000-0002-7585-2828

Benjamin Wenner @ https://orcid.org/0000-0002-2333-1297

Fernanda Batistel @ https://orcid.org/0000-0001-7313-7153