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ORIGINAL ARTICLE

Effect of partially replacing a barley-based concentrate with flaxseed-based products on the rumen bacterial population of lactating Holstein dairy cows

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Abstract

Aims: The effects of partial replacement of a barley-based concentrate with flaxseed-based products on the rumen bacterial population of lactating Holstein dairy cows were evaluated.
Methods and Results: Treatments fed were CONT, a normal diet that included barley silage, alfalfa hay and a barley-based concentrate that contained no flaxseed or faba beans; FLAX, inclusion of a nonextruded flaxseed-based product containing 55·0% flaxseed, 37·8% field peas and 6·9% alfalfa; EXT, similar to FLAX, but the product was extruded and EXTT, similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans. The rumen bacterial population was evaluated by utilizing 16S rRNA gene sequencing. Most abundant phyla, families and genera were unaffected. However, some taxa were affected; for example, unsaturated fatty acid content was negatively correlated with Clostridiaceae, and tannin content was negatively correlated with BS11 and Paraprevotellaceae.
Conclusions: Predominant rumen bacterial taxa were not affected, but the abundance of some taxa found in lower proportions shifted, possibly due to sensitivity to unsaturated fatty acids or tannins.
Significance and Impact of the Study: Flaxseed-based products were effective for partially replacing barley-based concentrate in rations of lactating dairy cows. No negative effects of these products were observed on the abundance of predominant rumen bacterial taxa, with only minor shifts in less abundant bacteria.

Introduction

Enriched unsaturated fatty acid animal products may offer beneficial effects to human health (Lee *et al.* 2005). Increasing the concentration of these fatty acids in ruminant products, however, is difficult due to intensive biohydrogenation by ruminal micro-organisms (Palmquist *et al.* 1993; Bauman and Griinari 2001; Jenkins *et al.* 2008). Thus, there has been significant interest to develop feeding strategies to decrease ruminal biohydrogenation of unsaturated fatty acids while ensuring high availability in the small intestine (Beam *et al.* 2000).

Supplementation with raw flaxseed or extruded flaxseed has been suggested to be an effective strategy to increase the availability of polyunsaturated fatty acids in the small intestine (Litton 2008; He *et al.* 2012). For example, extruded flaxseed increased the content of α-linolenic acid in blood serum or milk of dairy cattle (Kennelly 1996; Oeffner *et al.* 2013; Moats *et al.* 2015). Furthermore, the inclusion of dietary tannins in ruminant rations may be an effective approach for mitigating the biohydrogenation of polyunsaturated fatty acids (Vasta *et al.* 2009). However, dietary fat (Enjalbert *et al.* 2017) or tannins (Vasta *et al.* 2010) may have detrimental

effects on bacterial taxa within the rumen or may negatively affect animal production performance (Vasta *et al.* 2010). Nonetheless, most studies evaluating these dietary strategies for mitigating fatty acid biohydrogenation have not determined effects on the broad bacterial community structure of the rumen of lactating Holstein cows. Consequently, the impact of dietary unsaturated fatty acids, feed extrusion and tannins on the broad bacterial population remains poorly understood *in vivo*. This research gap can now be addressed using molecular techniques in combination with bioinformatics, which have enabled researchers to evaluate the impact of diets on bacterial community structure (Krause *et al.* 2013; Chaucheyras-Durand and Ossa 2014; Castillo-Lopez *et al.* 2017). For example, high-throughput DNA sequencing provides new insights into the broad bacterial population of the rumen (Callaway *et al.* 2010; Aldai *et al.* 2012; Castillo-Lopez *et al.* 2014).

In addition, the bacterial population of the rumen influences production performance (Myer *et al.* 2015), ruminal fermentation (Fernando *et al.* 2010; Anderson *et al.* 2016), ruminal pH and fermentation efficiency (Callaway *et al.* 2010), metabolizable protein supply (Castillo-Lopez *et al.* 2013) and milk composition in dairy cattle (Jami *et al.* 2014). Consequently, investigating the effects of diet composition and ingredients being generated by the dairy feeding industry on the rumen bacterial community is essential not only for improving milk yield and composition, but also for preventing negative impacts on rumen function. Therefore, the objective of this study was to evaluate the effect of partial replacement of a barley-based concentrate with different flaxseed-based products on the rumen bacterial community structure of lactating Holstein dairy cows, assessed with high-throughput DNA sequencing. Our hypothesis is that inclusion of flaxseed-based products in dairy rations will shift the abundance of ruminal bacteria.

Materials and methods

Animal care and housing, and experimental design

This experiment was conducted at the University of Saskatchewan Rayner Dairy Cattle Research and Teaching Facility (Saskatoon, Saskatchewan, Canada); it was performed in accordance with the guidelines published by the Canadian Council on Animal Care (1997). The protocols used in this study were preapproved by the University of Saskatchewan Animal Care and Use Committee (protocol number 20040048).

A total of eight multiparous, lactating Holstein cows from the University of Saskatchewan Greenbrae herd (mean and SD, 116·5 ± 17·5 DIM; 712·7 ± 92·3 kg BW) were used in a replicated 4 × 4 Latin square experimental design. Four of these cows were fitted with permanent ruminal cannulae to facilitate ruminal digesta sampling for microbial community analyses. Each experimental period comprised 28 days, which consisted of 26 days for dietary adaptation followed by 2 days for sample collection to provide enough time for animal adaptation to treatment change (Lillis *et al.* 2011; Boots *et al.* 2013). Cows were housed in individual tie stalls with continuous access to fresh water and feed except during milking. Animals were milked three times daily at 04:30, 12:30 and 19:00 h in a double six Herringbone parlour (DeLaval International, Peterborough, ON). The individual tie stalls were equipped with rubber mats. In addition, wood shavings were used for bedding and were replaced daily.

Experimental treatments, feed samples and feed chemical analysis

Rations were offered twice daily at 09:30 and 17:00 h for *ad libitum* access as total mixed rations. Each ration was mixed using a small-batch mixing cart (Data Ranger, American Calan, Northwood, NH). Treatments (DM basis; Table 1) were (i) CONT, a normal diet containing 28·1% barley silage, 20·0% alfalfa hay and 51·8% of a barley-based concentrate that contained no flaxseed or faba beans; (ii) FLAX, inclusion of 11·4% of a nonextruded flaxseed-based product which contained 55·0% flaxseed, 37·8% ground field peas and 6·9% dehydrated alfalfa; (iii) EXT, inclusion of 11·4% of an extruded flaxseed-based product which contained 55·0% flaxseed, 37·8% ground field peas and 6·9% dehydrated alfalfa and (iv) EXTT, inclusion of 11·4% of an extruded flaxseed-based product which contained 55·0% flaxseed, 37·8% ground high-tannin faba beans and 6·9% dehydrated alfalfa.

The barley-based concentrate was partially substituted with the inclusion of the corresponding flaxseed-based product in FLAX, EXT and EXTT, and these products included 0·4% of mould inhibitor plus vitamin E as antioxidant. High-tannin faba beans corresponded to variety Malik 9-4. Experimental diets were formulated based on two factors: (i) providing similar levels of net energy for lactation and (ii) achieving dietary ether extract levels approaching, but not exceeding 6% (DM basis) for the three flaxseed-containing treatments. All flaxseed-based products were manufactured and supplied by a local company (Oleet Processing Ltd., a division of O&T Farms Ltd., Regina, SK, Canada). Extruded flaxseed-based products were manufactured using a dry extrusion method with a single screw extruder (Model 2500; Insta-Pro International, Urbandale, IA) with barrel temperature averaging 120°C.

Table 1 Ingredient composition of the control and diets containing different flaxseed-based products fed to lactating Holstein dairy cows

Item	Treatment*			
	CONT	FLAX	EXT	EXTT
Ingredient, % DM				
Barley silage	28·1	28·1	28·1	28·1
Alfalfa hay	20·0	20·0	20·0	20·0
Ground corn grain	9·1	7·1	7·1	7·1
Pea grain	3·8	3·0	3·0	3·0
Ground barley grain	23·8	18·5	18·5	18·5
Canola meal solvent	3·8	3·0	3·0	3·0
Soybean meal solvent	4·3	3·3	3·3	3·3
Corn gluten meal	1·0	0·8	0·8	0·8
Corn distillers	1·7	1·3	1·3	1·3
Mineral and vitamin premix†	1·0	1·0	1·0	1·0
Palmitic acid	7·2	6·0	6·0	6·0
Molasses cane	7·5	5·9	5·9	5·9
Biotin‡	0·04	0·03	0·03	0·03
R-choline§	0·22	0·17	0·17	0·17
K-Mg-S	0·08	0·07	0·07	0·07
Sodium bicarbonate	0·53	0·41	0·41	0·41
Limestone	0·56	0·44	0·44	0·44
Niacin	0·02	0·02	0·02	0·02
Salt	0·23	0·18	0·18	0·18
Nonextruded flaxseed-based product	0	11·4	0	0
Extruded flaxseed-based product	0	0	11·4	0
Extruded flaxseed-based product with tannins	0	0	0	11·4

*CONT: a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX: inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT: similar to FLAX, but the product was extruded; EXTT: similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

†Mineral–vitamin premix contained (kg^{-1} DM): 160 g Ca, 70 g P, 70 g Mg, 20 g K, 12·6 g S, 100 g Cl; 1005 mg Fe, 1507 mg Mn, 2513 mg Zn, 80 mg I, 30 mg Co, 20 mg Se, 251, 256 IU vitamin A, 80, 402 IU of vitamin D3 and 2010 IU vitamin E.

‡Biotin: 20 g kg^{-1} DM biotin source.

§R-Choline: 250 g kg^{-1} DM choline source.

Samples of rations, barley silage, alfalfa hay, barley-based concentrate and flaxseed-based products were collected daily from day 21 to 28 of each period and pooled by treatment within each period. Feed samples were stored at −20°C pending analysis for chemical composition. In addition, barley silage samples were collected twice a week during the experiment for microwave DM determination (Valkeners *et al.* 2008). Briefly, a sample of approximately 100 g was heated in a microwave oven for 4 min. During the second step, drying time was decreased to 30 s, and the second step was repeated until obtaining a constant weight in two consecutive measurements. To avoid burning of the sample, a glass of water was also placed in the microwave. These DM data were used for adjusting the diet DM to ensure proper inclusion of ingredients in each treatment.

Alfalfa hay, barley silage and concentrate samples were dried at 55°C in a forced air oven for 48 h, and orts were freeze dried. Dried feed ingredients and orts were then ground to pass through a 1-mm screen (Christy-Norris mill, Christy and Norris Ltd., Chelmsford, UK) and analysed for chemical composition by an external laboratory (Cumberland Valley Analytical Services, Hagerstown, MD), which included DM (method no. 930.15; AOAC 2000), N (method no. 990.03; Leco FP-528 Nitrogen Combustion Analyzer; Leco Corp., St. Joseph, MI), NDF (Van Soest *et al.* 1991), starch (Hall 2009), ether extract using diethyl ether (method no. 2003.05; AOAC 2006) and ash (method no. 942.05; AOAC 2000). The nutrient composition of each total mixed ration (Table 2) was calculated based on analysis of individual ingredients, barley-based concentrate and flaxseed-based products and the rate of inclusion in the respective treatment. This method of reporting chemical composition of dairy diets is highly recommended, because when sampling total mixed rations for analysis of chemical composition results may be affected by sampling variation (Weiss *et al.* 2016). Feed fatty acid analysis was conducted at Lipid Analytical Services Ltd. (Guelph, ON, Canada); concentration of fatty acids was expressed as per cent of fatty acids methyl esters. The chemical analyses of individual feed ingredients were then used to calculate the chemical composition of experimental diets. In addition, feed samples were submitted to Lethbridge Research Centre (Lethbridge, AB) for determination of tannins using the acid–butanol assay (Porter *et al.* 1986).

Sampling of whole ruminal contents for bacterial community analysis

On days 27 and 28 of each experimental period, samples of intact, nonstrained ruminal contents (solid and liquid fractions) were taken using new palpation sleeves for each cow at each sampling time point. In order to obtain representative samples from the rumen of each cow, grab samples were taken from the caudal ventral sac, cranial ventral sac and two samples from the ruminal digesta mat in the dorsal rumen of each animal; samples were collected so that every 6-h interval in a 24-h period was represented. Specifically, these samples were collected at 10:00, 16:00 and 22:00 h on day 27, and 04:00 h on day 28. Within each time point, samples collected from the same cow were pooled, and a 10-ml subsample was

Table 2 Analysed chemical composition of the control and diets containing different flaxseed-based products fed to lactating Holstein dairy cows

	Treatment*			
Item	CONT	FLAX	EXT	EXTT
Chemical composition†				
Dry matter, % as-fed	91·3	91·4	91·7	91·8
Crude protein, % DM	16·3	16·8	16·8	16·9
Neutral detergent fibre, % DM	30·4	30·6	30·8	30·9
Starch, % DM	28·0	25·8	25·9	26·0
Nonfibre carbohydrates, % DM	41·6	38·8	39·6	39·1
Crude fat, % DM	3·2	5·8	5·7	5·7
Ash, % DM	9·6	9·0	9·0	9·0
NE_L, Mcal kg^{-1}	1·35	1·45	1·44	1·44
Condensed tannins, mg g^{-1}	ND	ND	ND	1·17
Fatty acids, % of total FAME				
C14:0	0·96	0·84	0·84	0·84
C14:1 cis9	0·03	0·03	0·03	0·03
C15:0	0·19	0·19	0·19	0·18
C16:0	32·8	29·0	28·9	28·9
C16:1 cis9	0·71	0·72	0·72	0·72
C18:0	5·18	4·80	4·82	4·82
C18:1	13·2	13·6	13·7	13·8
C18:2 n6	29·7	29·3	29·4	28·7
C18:3 n3	14·3	18·8	18·6	19·2
C20:0	0·48	0·47	0·47	0·47
C20:1 cis11	0·48	0·48	0·48	0·48
C22:0	0·70	0·71	0·69	0·70
C22:1	0·48	0·40	0·40	0·40
C24:0	0·49	0·49	0·50	0·49
C24:1	0·13	0·13	0·13	0·13
Total saturated	40·8	36·5	36·4	36·4
Total monounsaturated	15·03	15·39	15·46	15·56
Total polyunsaturated	43·9	48·1	48·0	47·9

ND, not detected.

*CONT: a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX: inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT: similar to FLAX, but the product was extruded; EXTT: similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

†Analysis conducted at Cumberland Valley Analytical Services, Hagerstown, MD.

placed in a sterile 15-ml vial and immediately snap frozen at −80°C. Thus, a total of 64 composited ruminal digesta content samples were collected during the trial (four cows × four time points × four experimental periods). To obtain digesta samples representative of a 24-h period, at the end of the experiment, these samples were pooled to obtain one sample per cow within each of the four periods as previously outlined and conducted by other researchers for samples collected from cattle for microbial community evaluations (Lillis *et al.* 2011; Boots *et al.* 2013); these samples were used for DNA extraction, sequencing and bacterial phylogenetic analysis.

DNA extraction and library preparation for microbial community analysis

Collected whole ruminal digesta samples were homogenized using a flame-sterilized spatula, and DNA was extracted and purified utilizing the MoBio PowerMag Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA; Whiteley *et al.* 2012; Paz *et al.* 2016; Xie *et al.* 2016) according to manufacturer's instructions at the Department of Animal Science of University of Nebraska-Lincoln. The concentration of DNA in each sample was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The quality of extracted DNA was verified by running the DNA samples on a 1·5% agarose gel. Subsequently, the V3 hypervariable region of the 16S rRNA gene from the bacterial communities was amplified using the polymerase chain reaction (PCR) technique and using the universal barcoded primers 341F and 518R as outlined by Whiteley *et al.* (2012) and Paz *et al.* (2016). The PCR reactions were performed in 20 μl volumes and contained 0·5 U of Terra DNA polymerase (Clontech Laboratories, Mountain view, CA), 200 nmol l^{-1} of each primer, 50 ng of nucleic acid template or nontemplate control, 10 μl of Terra PCR buffer and 6·5 μl of PCR water. The cycling conditions were an initial denaturation of 98°C for 3 min; followed by 25 cycles of 98°C for 30 s, 52°C for 30 s and 68°C for 40 s; and a final extension of 68°C for 4 min. The resulting amplicons from targeted hypervariable region have been shown to be effective for describing the rumen bacterial communities (Paz *et al.* 2016; Xie *et al.* 2016). The quality of the amplified DNA was verified by resolving on a 1·5% agarose gel. Amplicons from each sample were pooled in equal amounts using the epMotion M5073 automated system (Eppendorf, Hauppauge, NY) and the resulting pooled library was purified using the Pippin Prep kit (Sage Science, Beverly, MA) according to the manufacturer's instructions, and analysed according to the Bio Analyzer High Sensitive DNA kit (Agilent Technologies, Santa Clara, CA); then, DNA concentration was measured with a Qubit 2·0 fluorometer (Invitrogen, Carlsbad, CA) and the library was stored at −20°C for later analyses.

High-throughput DNA sequencing and bacterial phylogenetic analysis

The amplicon library was subjected to high-throughput DNA sequencing at the Department of Animal Science of University of Nebraska-Lincoln according to the protocol utilized by Paz *et al.* (2016) and Xie *et al.* (2016). Briefly, this method was conducted using the Ion Torrent Personal Genome Machine (PGM; Life Technologies,

Carlsbad, CA), and applying the Sequencing Kit v2 on a 316 chip according to the manufacturer's instructions; then, the high-quality DNA sequences were binned into their respective samples based on their barcodes. Specifics for the methods used for emulsion PCR, bead deposition and sequencing on the PGM were as described by the manufacturer. The rationale behind the sequencing direction is to minimize sequencing errors. Because there is slightly more sequence variability towards the 518 end compared to the 341 end (Vasileiadis *et al.* 2012), and because the beginning of sequencing has less errors, sequencing started from the 518R end and moved towards the 341F end (which has less variability). Thus, there was less chance of sequencing errors on the region of the read which has more variability. Paired-end amplicons were not generated during this sequencing run; therefore, only single read sequences were generated and no contig assembly was performed.

Sequenced data were deposited in the NCBI Sequence Read Archive under the accession no. SRR6023841. Sequence reads were analysed using published bioinformatics pipelines UPARSE (drive5.com/uparse/; Edgar *et al.* 2011), QIIME (qiime.org; Caporaso *et al.* 2010) and MOTHUR (Schloss *et al.* 2009). Initial quality control of the generated sequences was performed using the Torrent Suite Software ver. 3.6.2 as outlined by Paz *et al.* (2016), which included trimming of the 3′ end of sequences that dropped below the average Q15 score over a 30-bp window and removing sequences with unidentified bases (N). Resulting sequences were downloaded from the Torrent Suite and demultiplexed using the QIIME software package (ver. 1.9.1) (Caporaso *et al.* 2010). During demultiplexing, sequences with an average quality score <25 were removed. Following demultiplexing, universal primers used for sequencing were removed, allowing one mismatch in the 5′ (518R) primer and two in the 3′ reverse primer (341F). Sequences shorter than 130 bp were removed and remaining sequences were trimmed to a fixed length of 130 bp (Paz *et al.* 2016). Quality trimmed sequences were then reverse complemented, screened for chimeric sequences using UCHIME (Edgar *et al.* 2011), and preclustered using the pseudo-single linkage-clustering algorithm to remove reads that resulted from sequencing errors (Huse *et al.* 2010). These sequences were then assigned to operational taxonomic units (OTUs) at 97% similarity using UPARSE pipeline (drive5.com/uparse/; Edgar 2011). Sequences from each OTU were then subjected to taxonomic classification using the latest version of the Greengenes taxonomy database (gg_13_5) (Wang *et al.* 2007). Based on the taxonomy information, any sequences associated with chloroplasts (from plant origin, and thus, most likely from feed) were filtered and discarded. In addition, representative OTU sequences were aligned to the bacterial 16S rRNA gene using the RDP aligner tool available at Michigan State University (https://rdp.cme.msu.edu/tutorials/aligner/RDPtutorial_ALIGNER.html); and those sequences that did not align with the sequenced region were filtered, thus removing OTU sequences that did not align within the expected region.

The OTU table was rarefied across samples to the lowest sample depth (6295 reads) using QIIME. All statistical analyses were performed with samples at an even depth. Furthermore, beta-diversity plots were generated in QIIME to evaluate differences based on sequence similarities and these plots were visualized with the Emperor visualization programme (Vazquez-Baeza *et al.* 2013). Moreover, alpha diversity estimators (Chao1 and observed species) and diversity index (Shannon) were evaluated for the overall community using QIIME (Caporaso *et al.* 2010). Good's coverage test was performed to evaluate if adequate sampling depth was achieved. A Venn diagram was constructed to illustrate the relationship and OTU distribution among treatments. To do so, the venn function in the gplots package of R was used (Warnes *et al.* 2015).

A core microbiome, which was defined as those OTUs present in all animals, was calculated. From the core microbiome, taxa were summarized and plots were generated at the taxonomic levels of phylum, class, order, family and genus, with emphasis on representative OTUs that represented at least 0·1% of the microbial community in each sample. To minimize animal to animal variation and to represent the shared OTUs within each diet, the core microbiome was analysed. This allows identification of the microbial community influenced by the treatment sorting through animal to animal variation. The hypothesis is that, if the treatment affects the microbial community, this effect should be present across multiple animals on the same treatment. Therefore, the analysis of the core microbiome allows identification of the effects that might otherwise be hidden in the data (Benson *et al.* 2010; Castillo-Lopez *et al.* 2014, 2017).

Compared to other sequencing platforms such as 454 Roche pyrosequencing (Castillo-Lopez *et al.* 2014) or Illumina (Castillo-Lopez *et al.* 2017), the use of ion torrent may have some limitations due to sequencing errors (Frey *et al.* 2014; Salipante *et al.* 2014). However, the quality control steps outlined by previous researchers such as initial quality control using the ion Torrent Suite Software, screening for chimeric sequences and preclustering using the pseudo-single linkage-clustering algorithm were specifically aimed at removing erroneous reads. Moreover, the core microbiota analysis where only OTUs that were present in all animals were considered should also filter out many OTUs generated due to

random sequencing errors as these would not be expected to occur in all animals.

Statistical analysis

Data collected on the abundance of bacterial phyla, families and genera, and sample bacterial richness (Chao1 and observed species) as well as diversity index (Shannon) were analysed using the MIXED procedure of SAS (ver. 9.1; SAS Institute Inc., Cary, NC). Fixed effects included the treatment and period, with cow as the random effect. The statistical model for this experiment was as follows:

$$Y_{ijk} = \mu + \beta_i + \rho_j + \alpha_k + \varepsilon_{ijk}$$

where Y_{ijk} represents observation ijk, μ represents the overall mean, β_i represents the random effect of cow i, ρ_j represents the fixed effect of period j and α_k represents the fixed effect of treatment k. The residual term ε_{ijk} was assumed to be normally, independently and identically distributed, with variance σ_e^2. The comparison of treatment means was conducted using the PDIFF option in the LSMEANS statement. In addition, using the CONTRAST statement, CONT was compared to FLAX+EXT +EXTT, and CONT was compared to each of the other treatments. Furthermore, FLAX was compared to EXT, and EXT was compared to EXTT. Treatment means are presented as least squares means. The largest standard error of the mean (SEM) is reported. Statistical significance was declared when $P \leq 0{\cdot}05$ and tendencies were discussed when $P > 0{\cdot}05$ and $\leq 0{\cdot}10$. The Spearman's correlation analysis was conducted to evaluate associations between dietary composition (content of fat, unsaturated fatty acids and tannins) and bacterial families and genera.

In addition, bacterial community composition differences were evaluated using the weighted UniFrac distance matrix as an input for a permutational multivariate analysis of variance (PERMANOVA) in R using the vegan package (adonis function) (Oksanen *et al.* 2015), where treatment was used as main effect.

Results

Diets, milk yield and composition

The inclusion of the flaxseed-based products in diets resulted in significant changes ($P < 0{\cdot}05$) in chemical composition; the per cent of unsaturated fatty acids increased by approximately 4%; replacement of field peas with high tannin faba beans resulted in a content of condensed tannins of 1·17 mg g^{-1} in EXTT. It is important to note that the level of condensed tannins decreased by 83% after extrusion (from 6·87 to 1·17 mg g^{-1}). Condensed tannins were not detected in CONT, FLAX or EXT, as was anticipated based on the ingredient composition of the flaxseed supplements. Production performance data and milk fatty acid composition have been reported (Moats *et al.* 2015). Briefly, there was a significant decrease ($P < 0{\cdot}05$) in DMI when EXT was fed compared to CONT (25·9 and 23·4 kg, respectively); fat corrected milk, however, was not affected and averaged 40·5 kg. In addition, significant changes ($P < 0{\cdot}05$) were observed in milk fatty acid profile; for example, when the flaxseed-based products were fed, the proportion of total saturated fatty acids decreased by 10·8%, the proportion of total polyunsaturated fatty acids increased by 0·60% and the concentration of Omega-3 increased by 0·51%. Nonetheless, no significant effects of treatment were observed on ruminal pH (6·03), ruminal digestibility of dry matter (38·4%), organic matter (40·7%) and neutral detergent fibre (35·7%).

Number of sequences, sample richness, diversity index and OTU distribution

Collectively, a total of 356 709 high-quality DNA sequences were obtained after initial quality control and filtering, and were used for downstream analysis. Diversity metric Chao1 was not affected by the inclusion of the flaxseed-based products ($P \geq 0{\cdot}38$); observed species ($P \geq 0{\cdot}34$) and the Shannon diversity index ($P \geq 0{\cdot}23$) remained unaffected as well (Table S1). The Good's coverage test showed that sequencing depth was able to characterize >98% of the bacterial community.

The Venn diagram for OTU distribution revealed that each treatment showed a number of unique OTUs. However, there were 1285 OTUs shared by the four diets, which represented 69·16% of total OTUs detected. In addition, according to the beta diversity for sequence similarities (Fig. S1) based on principal coordinate analysis, there appear to be two clusters and two sample outliers; however, no apparent clustering of microbial communities by treatment was found, indicating a similar spatial sample heterogeneity among the diets. The bacterial community analysis using PERMANOVA did not display a significant ($P = 0{\cdot}20$) effect on bacterial community composition.

Effects of diets on ruminal bacterial community composition

Bacterial phylogenetic analyses revealed the presence of 11 major phyla in the ruminal microbiome (Table 3). No significant shifts due to treatment were observed on most of these phyla. Predominant bacterial phyla were not affected and were Bacteroidetes ($P \geq 0{\cdot}24$),

Table 3 Effect of partially replacing a barley-based concentrate with different flaxseed-based products in dairy rations on the abundance of major bacterial phyla in the rumen of lactating Holstein cattle

	Treatment*					*P*-values‡					
Phylum, % of total	CONT	FLAX	EXT	EXTT	SEM†	1	2	3	4	5	6
Bacteroidetes	50·95	50·81	48·13	46·81	3·370	0·46	0·90	0·63	0·24	0·74	0·52
Firmicutes	43·97	44·93	45·55	48·97	3·734	0·48	0·83	0·73	0·26	0·90	0·47
Proteobacteria	1·30	1·07	1·50	1·64	0·274	0·69	0·42	0·46	0·30	0·18	0·84
Tenericutes	0·93	0·92	1·24	0·65	0·364	0·75	0·98	0·35	0·73	0·39	0·24
Verrucomicrobia	0·70	0·68	0·81	1·04	0·284	0·20	0·68	0·37	0·07	0·61	0·36
Spirochaetes	0·35	0·19	0·20	0·18	0·102	0·14	0·24	0·28	0·16	0·92	0·74
TM7	0·32	0·25	0·27	0·21	0·096	0·48	0·52	0·74	0·47	0·76	0·73
Fibrobacteres	0·30	0·12	0·55	0·26	0·263	0·88	0·55	0·42	0·51	0·22	0·20
Actinobacteria	0·22	0·24	0·22	0·29	0·048	0·85	0·76	0·81	0·69	0·63	0·56
SR1	0·15	0·27	0·07	0·06	0·057	0·64	0·03	0·08	0·07	<0·01	0·83
WPS2	0·08	0·10	0·10	0·12	0·036	0·16	0·13	0·36	<0·01	0·46	0·87
Other	0·72	0·68	0·56	0·52	0·118	0·36	0·65	0·35	0·42	0·61	0·78
Bacteroidetes/Firmicutes	1·15	1·13	1·05	0·95	0·110	0·47	0·81	0·79	0·22	0·97	0·37

*CONT: a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX: inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT: similar to FLAX, but the product was extruded; EXTT: similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

†The largest standard error of the mean is reported.

‡1: CONT *vs* FLAX+EXT+EXTT; 2: CONT *vs* FLAX; 3: CONT *vs* EXT; 4: CONT *vs* EXTT; 5: FLAX *vs* EXT; 6: EXT *vs* EXTT.

Firmicutes ($P \geq 0{\cdot}26$), Proteobacteria ($P \geq 0{\cdot}18$), Tenericutes ($P \geq 0{\cdot}24$) and Spirochaetes ($P \geq 0{\cdot}14$). However, compared to CONT, Verrucomicrobia tended ($P = 0{\cdot}07$) to increase for EXTT. In addition, compared to CONT, the abundance of the phylum SR1 was greater ($P < 0{\cdot}05$) for FLAX, tended to be lower for EXT ($P = 0{\cdot}08$) and for EXTT ($P = 0{\cdot}07$); and compared to FLAX, SR1 was lower ($P < 0{\cdot}01$) for EXT. Furthermore, compared to CONT, the phylum WPS2 increased ($P < 0{\cdot}01$) for EXTT. The ratio of Bacteroidetes to Firmicutes was not affected ($P \geq 0{\cdot}22$) by treatment and averaged $1{\cdot}10 \pm 0{\cdot}100$.

Table 4 lists major bacterial families detected. No changes were observed on the abundance of the bacterial families Prevotellaceae ($P \geq 0{\cdot}23$), unclassified Bacteroidales ($P \geq 0{\cdot}18$), Veillonellaceae ($P \geq 0{\cdot}28$), Lachnospiraceae ($P \geq 0{\cdot}55$), unclassified Clostridiales ($P \geq 0{\cdot}26$) and Ruminococcaceae ($P \geq 022$). However, variations due to treatment were observed in the abundance of some bacterial families. For example, compared to CONT, the abundance of the family S24-7 increased ($P < 0{\cdot}05$), BS11 decreased ($P < 0{\cdot}05$), Clostridiaceae decreased ($P = 0{\cdot}04$) and Christensenellaceae decreased ($P < 0{\cdot}05$) when treatments included any of the flaxseed-based products. In addition, compared to CONT, the abundance of S24-7 ($P < 0{\cdot}01$) increased, and Clostridiaceae tended ($P = 0{\cdot}06$) to increase for FLAX. Furthermore, compared to CONT, the abundance of BS11 ($P < 0{\cdot}05$) decreased, Mogibacteriaceae tended ($P = 0{\cdot}06$) to decrease, Clostridiaceae ($P = 0{\cdot}05$) decreased and Christensenellaceae decreased ($P < 0{\cdot}05$) for EXT. Moreover, compared to CONT, BS11 decreased ($P < 0{\cdot}05$), Paraprevotellaceae decreased ($P = 0{\cdot}05$), RFP12 tended ($P = 0{\cdot}09$) to increase, Clostridiaceae tended to decrease ($P = 0{\cdot}09$), Christensenellaceae decreased ($P = 0{\cdot}01$) and WCHB1-25 increased ($P = 0{\cdot}01$) for EXTT. In addition, compared to FLAX, S24-7 ($P < 0{\cdot}01$) decreased, BS11 tended ($P = 0{\cdot}09$) to decrease, Mogibacteriaceae ($P < 0{\cdot}05$) decreased and Succinivibrionaceae ($P < 0{\cdot}05$) increased for EXT.

Table 5 lists predominant bacterial genera detected. No effect of diet was detected on major genera and were *Prevotella* ($P \geq 0{\cdot}10$), unclassified Bacteroidales ($P \geq 0{\cdot}26$), *Succiniclasticum* ($P \geq 0{\cdot}30$), unclassified Clostridiales ($P \geq 0{\cdot}32$), unclassified Ruminococcaceae ($P \geq 0{\cdot}37$) and *Butyrivibrio* ($P \geq 0{\cdot}25$). However, compared to CONT, an unclassified bacterial genus belonging to S24-7 increased ($P < 0{\cdot}05$), an unclassified BS11 decreased ($P = 0{\cdot}04$), YRC22 ($P = 0{\cdot}05$) decreased, CF231 ($P < 0{\cdot}01$) decreased and *Clostridium* ($P < 0{\cdot}01$) decreased when treatments included any of the flaxseed-based products. In addition, compared to CONT, an unclassified S24-7 ($P < 0{\cdot}01$) increased, CF231 ($P < 0{\cdot}05$) decreased, *Clostridium* ($P < 0{\cdot}01$) decreased and *Coprococcus* tended ($P = 0{\cdot}08$) to increase for FLAX. Furthermore, compared to CONT, an unclassified genus belonging to BS11 ($P < 0{\cdot}05$) decreased, YRC22 ($P = 0{\cdot}05$) decreased, CF231 ($P < 0{\cdot}05$) decreased, *Clostridium* ($P < 0{\cdot}01$) decreased and *Oscillospira* tended ($P = 0{\cdot}08$) to increased

Table 4 Effect of partially replacing a barley-based concentrate with different flaxseed-based products in dairy rations on the abundance of major bacterial families in the rumen of lactating Holstein cattle

	Treatment*					*P*-values‡					
Family, % of total	CONT	FLAX	EXT	EXTT	SEM†	1	2	3	4	5	6
Prevotellaceae	20·25	18·12	23·38	19·82	2·621	0·94	0·56	0·40	0·89	0·23	0·35
Veillonellaceae	13·72	15·50	15·16	20·10	5·859	0·51	0·77	0·82	0·28	0·96	0·45
Unclassified Bacteroidales	13·47	12·94	10·27	11·77	1·874	0·29	0·80	0·18	0·38	0·28	0·49
Lachnospiraceae	12·15	11·94	12·64	11·85	1·186	0·99	0·87	0·70	0·79	0·62	0·55
S24-7	11·57	14·59	12·17	11·90	0·708	0·02	<0·01	0·24	0·43	<0·01	0·56
Unclassified Clostridiales	9·99	9·96	9·15	8·55	1·217	0·54	0·90	0·64	0·26	0·58	0·56
Ruminococcaceae	6·10	5·57	6·50	6·42	0·607	0·88	0·43	0·54	0·57	0·22	0·90
BS11	3·77	3·17	1·30	1·22	0·809	0·04	0·49	0·04	0·02	0·09	0·92
Paraprevotellaceae	1·62	1·59	1·27	1·27	0·149	0·26	0·71	0·83	0·05	0·88	0·10
Desulfovibrionaceae	1·02	1·06	1·32	1·25	0·368	0·63	0·93	0·56	0·63	0·64	0·87
Erysipelotrichaceae	1·00	0·77	1·03	1·30	0·228	0·82	0·34	0·87	0·17	0·30	0·28
Unclassified RF39	0·92	0·92	1·29	0·80	0·383	0·79	0·99	0·39	0·73	0·42	0·27
Mogibacteriaceae	0·57	0·63	0·48	0·55	0·069	0·51	0·17	0·06	0·46	0·02	0·13
RFP12	0·47	0·50	0·54	0·70	0·162	0·30	0·81	0·60	0·09	0·78	0·26
Clostridiaceae	0·40	0·26	0·25	0·30	0·062	0·04	0·06	0·05	0·09	0·83	0·44
Spirochaetaceae	0·32	0·20	0·20	0·17	0·088	0·18	0·32	0·32	0·20	0·99	0·83
F16	0·32	0·25	0·28	0·25	0·089	0·48	0·52	0·74	0·47	0·76	0·73
Fibrobacteriaceae	0·30	0·14	0·51	0·15	0·257	0·88	0·55	0·42	0·51	0·22	0·20
Coriobacteriaceae	0·22	0·20	0·20	0·20	0·039	0·63	0·74	0·74	0·61	0·99	0·88
Christensenellaceae	0·17	0·12	0·07	0·05	0·038	0·02	0·18	0·03	0·01	0·25	0·62
Bacteroidaceae	0·10	0·12	0·16	0·08	0·040	0·61	0·65	0·26	0·56	0·48	0·13
WCHB1-25	0·08	0·06	0·15	0·27	0·068	0·12	0·77	0·25	0·01	0·19	0·12
Succinivibrionaceae	0·08	0·01	0·16	0·10	0·040	0·70	0·26	0·15	0·62	0·04	0·26

*CONT: a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX: inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT: similar to FLAX, but the product was extruded; EXTT: similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

†The largest standard error of the mean is reported.

‡1: CONT *vs* FLAX+EXT+EXTT; 2: CONT *vs* FLAX; 3: CONT *vs* EXT; 4: CONT *vs* EXTT; 5: FLAX *vs* EXT; 6: EXT *vs* EXTT.

for EXT. Furthermore, compared to CONT, an unclassified genus belonging to BS11 ($P < 0·05$) decreased, YRC22 ($P < 0·05$) decreased, CF231 ($P < 0·01$) decreased, an unclassified RFP12 tended ($P = 0·09$) to increase and *Clostridium* ($P < 0·01$) decreased for EXTT. Moreover, compared to FLAX, unclassified genus belonging to S24-7 ($P < 0·01$) decreased, unclassified BS11 tended ($P = 0·09$) to decrease, YRC22 tended ($P = 0·09$) to decrease, *Coprococcus* ($P < 0·05$) decreased and *Oscillospira* ($P < 0·05$) increased for EXT.

Correlation coefficients

Correlation analysis between dietary components (content of fat, unsaturated fatty acids and tannins) and bacterial families and genera (Table 6) revealed that the content of dietary fat tended ($P \leq 0·09$) to be negatively correlated with the abundance of the families BS11, Christensenellaceae and Clostridiaceae, and was negatively correlated ($P < 0·05$) with the genus *Coprococcus*, but was positively correlated with unclassified Veillonellaceae ($P = 0·02$). Dietary unsaturated fatty acid content tended ($P = 0·07$) to be negatively correlated with the family Clostridiaceae, but was positively correlated ($P \leq 0·04$) with the genera unclassified Veillonellaceae and *Schwartzia*. Dietary tannin content tended ($P \leq 0·08$) to be negatively correlated with the families BS11, Paraprevotellaceae and Christensenellaceae, but was positively correlated ($P < 0·01$) with WCHB1-25; in addition, tannin content tended to be negatively correlated ($P = 0·09$) with the genus *Prevotella*, but was positively correlated ($P \leq 0·02$) with *Oscillospira* and *Bulleidia*.

Discussion

The gut microbial population influences physiology, metabolism, nutrition and immune function with disruption of this community being linked to gastrointestinal conditions (Guinane and Cotter 2013; Ridaura *et al.* 2013). In ruminants, gut microbes represent a source of metabolizable protein (Spicer *et al.* 1986; NRC 2000), they play an essential role in volatile fatty acid

Table 5 Effect of partially replacing a barley-based concentrate with different flaxseed-based products in dairy rations on the abundance of major bacterial genera in the rumen of lactating Holstein cattle

	Treatment*					*P*-values‡					
Genus, % of total	CONT	FLAX	EXT	EXTT	SEM†	1	2	3	4	5	6
Prevotella	20·20	14·77	22·53	19·80	2·633	0·67	0·18	0·53	0·90	0·10	0·46
Unclassified Bacteroidales	13·50	9·87	10·06	11·77	2·554	0·28	0·26	0·33	0·57	0·95	0·61
Succiniclasticum	12·15	13·62	13·24	18·22	5·847	0·55	0·81	0·86	0·30	0·95	0·45
Unclassified S24-7	11·57	14·59	12·17	11·90	0·743	0·02	<0·01	0·24	0·43	<0·01	0·56
Unclassified Clostridiales	9·80	7·57	9·00	8·35	1·932	0·42	0·32	0·74	0·51	0·56	0·78
Butyrivibrio	7·37	7·07	7·90	7·08	0·917	0·96	0·65	0·44	0·61	0·27	0·25
Unclassified Ruminococcaceae	3·93	3·56	4·08	3·92	0·522	0·86	0·50	0·75	0·99	0·37	0·75
Unclassified Lachnospiraceae	3·92	3·17	4·09	4·02	0·760	0·83	0·44	0·86	0·91	0·39	0·94
Unclassified BS11	3·77	3·19	1·30	1·22	0·809	0·04	0·49	0·03	0·02	0·09	0·92
Ruminococcus	1·95	1·86	2·00	2·17	0·115	0·62	0·60	0·80	0·17	0·49	0·28
YRC22	1·05	1·02	0·86	0·80	0·134	0·05	0·78	0·05	0·01	0·09	0·39
Desulfovibrio	0·90	0·89	1·00	1·22	0·282	0·50	0·99	0·69	0·22	0·70	0·45
CF231	0·57	0·21	0·30	0·15	0·069	<0·01	0·01	0·03	<0·01	0·40	0·11
Unclassified Veillonellaceae	0·47	0·66	0·60	0·57	0·084	0·12	0·11	0·25	0·29	0·57	0·79
Unclassified RFP12	0·47	0·50	0·54	0·70	0·162	0·30	0·81	0·60	0·09	0·78	0·26
P-75-A5	0·40	0·37	0·47	0·60	0·163	0·43	0·82	0·59	0·13	0·48	0·35
Clostridium	0·40	0·13	0·16	0·20	0·054	<0·01	<0·01	<0·01	<0·01	0·62	0·42
Selenomonas	0·45	0·27	0·43	0·45	0·167	0·72	0·46	0·95	0·99	0·53	0·95
Schwartzia	0·37	0·43	0·43	0·45	0·131	0·55	0·67	0·67	0·54	0·99	0·90
Bulleidia	0·35	0·29	0·32	0·40	0·095	0·87	0·58	0·82	0·62	0·76	0·51
Treponema	0·32	0·20	0·20	0·18	0·088	0·18	0·32	0·32	0·20	0·99	0·83
Fibrobacter	0·30	0·14	0·51	0·15	0·257	0·88	0·55	0·42	0·51	0·22	0·20
Anaerovibrio	0·30	0·43	0·42	0·47	0·088	0·17	0·30	0·34	0·16	0·92	0·66
Coprococcus	0·25	0·31	0·22	0·20	0·021	0·88	0·08	0·44	0·11	0·04	0·39
Oscillospira	0·22	0·18	0·32	0·25	0·041	0·47	0·34	0·08	0·55	0·03	0·16
Unclassified Coriobacteriaceae	0·20	0·21	0·18	0·20	0·044	0·94	0·82	0·70	0·99	0·59	0·70
Lachnospira	0·17	0·16	0·12	0·10	0·033	0·20	0·69	0·26	0·13	0·48	0·69
Moryella	0·17	0·10	0·14	0·18	0·042	0·30	0·12	0·52	0·99	0·30	0·52
Shuttleworthia	0·17	0·14	0·24	0·08	0·097	0·76	0·72	0·60	0·34	0·43	0·20
L7A-E11	0·15	0·13	0·18	0·27	0·064	0·49	0·76	0·74	0·13	0·57	0·25
Anaerostipes	0·10	0·09	0·13	0·13	0·037	0·67	0·90	0·55	0·59	0·52	0·90
Mogibacterium	0·08	0·12	0·04	0·15	0·052	0·51	0·46	0·52	0·15	0·23	0·09
Unclassified WCHB1-25	0·08	0·13	0·10	0·28	0·085	0·33	0·66	0·81	0·11	0·84	0·19

*CONT: a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX: inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT: similar to FLAX, but the product was extruded; EXTT: similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

†The largest standard error of the mean is reported.

‡1: CONT *vs* FLAX+EXT+EXTT; 2: CONT *vs* FLAX; 3: CONT *vs* EXT; 4: CONT *vs* EXTT; 5: FLAX *vs* EXT; 6: EXT *vs* EXTT.

production and feed digestion (McAllister *et al.* 1994) and milk composition (Jami *et al.* 2014). Moreover, the bacterial community is responsible for fatty acid biohydrogenation (Jenkins *et al.* 2008). Therefore, to effectively develop feeding strategies to enhance production performance or quality of dairy products, researchers must understand the effects of diet composition or biohydrogenation mitigating strategies on the broad ruminal microbiome *in vivo*.

Given the laborious nature of studies involving the evaluation of ruminal fermentation and the rumen microbial community, some of the experiments have been conducted using small number of animals (Lillis *et al.* 2011; Boots *et al.* 2013; Mohammed *et al.* 2014; Denman *et al.* 2015) through the Latin square design, which is commonly used in cattle nutrition studies (Lillis *et al.* 2011; Boots *et al.* 2013), mostly because it is efficient as it generates replication with limited experimental units. However, it should be noted that the potential for carryover effects is one limitation of the design. Especially when evaluating the effects of plant secondary compounds on the microbial community because of their effect on animal physiology and metabolism (Dearing *et al.* 2005). In addition, once ruminal micro-organisms

Table 6 Correlation coefficients between the abundance of bacterial families and genera in the rumen and the content of fat, unsaturated fatty acids and tannins in diets* fed to lactating Holstein dairy cattle

Dietary component	Bacterial family or genus	Correlation coefficient (*r*)	*P*-value
Fat, %	BS11	−0·5118	0·06
	Christensenellaceae	−0·4835	0·07
	Clostridiaceae	−0·4670	0·09
	Coprococcus	−0·8001	0·01
	Unclassified Veillonellaceae	0·8000	0·02
	Schwartzia	0·6537	0·07
Unsaturated fatty acids, %	Clostridiaceae	−0·4863	0·07
	Unclassified Veillonellaceae	0·7746	0·02
	Schwartzia	0·7089	0·04
Tannins, mg g^{-1}	BS11	−0·5115	0·06
	Paraprevotellaceae	−0·5763	0·03
	Christensenellaceae	−0·4810	0·08
	WCHB1-25	0·6619	<0·01
	Prevotella	−0·6299	0·09
	Oscillospira	0·7604	0·02
	Bulleidia	0·8001	0·01

*CONT: a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX: inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT: similar to FLAX, but the product was extruded; EXTT: similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

have been treated with adverse plant dietary products, they may be no longer naïve and their reaction may be damped on successive treatments (Kohl and Dearing 2016). In this study, the experiment was designed with 28-day periods as an attempt to minimize potential carryover effects, with the first 26 days serving as a 'washout' period and the final 2 days serving for collection of ruminal digesta for bacterial community analysis.

Increasing the size of the study would improve the experiential precision (Stroup 1999; Kononoff and Hanford 2006). However, despite being relatively small, the use of the Latin square experimental design in this study allowed detection of important and statistically significant differences in rumen fermentation and microbial analysis, as in previous reports using the same design (Lillis *et al.* 2011; Boots *et al.* 2013; Ramirez Ramirez *et al.* 2016a,b).

Feeding flaxseed or flaxseed-based products to dairy cows and effects on the overall rumen bacterial community

Feeding flaxseed has been shown to improve milk fatty acid profile without affecting milk production (Oeffner *et al.* 2013). Current advances in the dairy feeding industry is spurring development of new flaxseed-based feed ingredients to enhance milk fatty acid profile; and the effects of those products on the broad rumen bacterial community of dairy cows must be clearly elucidated. Regardless of treatment, predominant ruminal bacteria agree with previous reports (Petri *et al.* 2012) showing that major bacterial phyla in the rumen of cattle are Bacteroidetes, Firmicutes and Proteobacteria. Collectively, these phyla represented approximately 96% of the rumen bacterial community in the current study. The influence of diet on the diversity and community composition of ruminal contents has long been recognized (Tajima *et al.* 2001; Fernando *et al.* 2010). In this experiment, when compared to the normal diet, the inclusion of raw flaxseed or extruded flaxseed with or without high-tannin faba beans did not cause drastic shifts on the abundance of major ruminal bacterial phyla. Contrasting these observations, Kong *et al.* (2010) used quantitative fluorescence *in situ* hybridization and found that inclusion of flaxseed reduced the total abundance of the phyla Bacteroidetes, Firmicutes and Proteobacteria in the rumen of cows fed silage-based diets. The discrepancies between these observations may be related to the chemical composition of the diets and the available substrates for microbial growth. In our study, the main dietary changes involved the increase in ether extract and polyunsaturated fatty acids in diets with flaxseed inclusion; in addition, there was a change in physical processing of flaxseed among diets, with a constant forage base. Thus, substrate availability for bacterial fermentation was relatively similar across treatments suggesting that bacterial phyla and other major taxa distribution may be resilient to changes in physical form of feeds when dietary fat does not exceed 6% in the diet.

Taxonomic analyses at the family and genus levels agree with previous findings using DNA pyrosequencing indicating that the ruminal microbiome is largely composed of the bacterial families Prevotellaceae, Lachnospiraceae and Ruminococcaceae and the genera *Prevotella*, *Succiniclasticum* and *Ruminococcus* (Castillo-Lopez *et al.* 2014). *Prevotella*, the largest bacterial genus detected, is composed of versatile organisms that can utilize a variety of nutrients including protein, starch, pectins and hemicellulose (Russell 2002), and has also been reported to predominate in the rumen of cattle being fed forage-based diets supplemented with corn distillers grains (Ramirez Ramirez *et al.* 2016a,b). In agreement with Kong *et al.* (2010), this experiment indicated that the fibre digesting genus *Fibrobacter* accounted for a minor fraction of the bacterial communities across diets. Overall, inclusion of flaxseed-based products did not affect predominant bacterial families and genera, and only affected taxa found in lower proportions, which could partially explain the lack of negative effects on

ruminal digestibility of DM, organic matter and neutral detergent fibre.

Effect of unsaturated fatty acids, extrusion and dietary tannins on rumen bacterial community structure and function

Dietary strategies to improve fatty acid profile of ruminant products have included feeding unsaturated fatty acids, feed extrusion or supplementation with tannins. Although effects on some bacterial taxa (Vasta *et al.* 2010; Enjalbert *et al.* 2017) or production performance has been acknowledged, the impact of such strategies on the broad ruminal bacterial communities of dairy cows *in vivo* is yet to be clearly elucidated.

Ruminal bacteria, specially fibrolytic bacteria, may be negatively affected by dietary fat (Maia *et al.* 2006; Enjalbert *et al.* 2017). For example, Maia *et al.* (2010) reported that unsaturated fatty acids decreased the abundance of *Butyrivibrio fibrisolvens in vitro*. In addition, negative effects of dietary linseed oil have been reported on the genera *Fibrobacter*, *Prevotella* and *Ruminococcus* (Huws *et al.* 2014; Enjalbert *et al.* 2017). In the present experiment, the 4% increment in dietary unsaturated fatty acids was not accompanied by a decrease in the abundance of these genera. This may indicate that the increase in dietary fat and unsaturated fatty acids was not severe enough to exert negatively impacts on those taxa; in agreement with this observation, no negative effects were detected on ruminal fibre digestion across treatments (Moats *et al.* 2015).

Feed extrusion has been applied to decrease fatty acid saturation because heat denatures the protein matrix surrounding the fat droplets, consequently reducing the access of ruminal bacteria to dietary fat (Kennelly 1996). Moreover, Vasta *et al.* (2007) and Schofield *et al.* (2001) suggested that tannins may inhibit the activity of biohydrogenating bacteria because tannins can interfere with bacterial growth. Within the bacterial population residing in the rumen, a number of bacteria that participate in fatty acid saturation have been identified, which include bacteria belonging to the genera *Pseudobutyrivibrio* (Paillard *et al.* 2007), *Propionibacterium* (McKain *et al.* 2010), *Clostridium*, *Butyrivibrio* (Polan *et al.* 1964; Castro-Carrera *et al.* 2014), *Selenomonas* (Fujimoto *et al.* 1993) and *Lactobacillus* (Jenkins *et al.* 2008; Sakurama *et al.* 2014). In the present experiment, the abundance of the genera *Selenomonas* and *Butyrivibrio* were similar across treatments. However, Buccioni *et al.* (2014) and Vasta *et al.* (2010) reported an increase in *B. fibrisolvens* and a decrease in *Butyrivibrio proteoclasticus* in the rumen of sheep supplemented with quebracho tannins. This suggests that biohydrogenating bacterial species within the same genus show different degrees of sensitivity to dietary tannins (Nelson *et al.* 1997; Schofield *et al.* 2001). In this study, we did not evaluate bacterial species; however, it is possible that the lack of an effect of EXTT on most bacterial taxa may have been due to the lower tannin concentration compared to Vasta *et al.* (2010) who utilized quebracho-supplemented diets containing 6·4% tannins. It is important to note that the content of tannins in the extruded product containing high-tannin faba beans was lower than expected, the 83% decrease in tannin content of the extruded product may have been caused by high temperature during the extrusion process (Iram *et al.* 2014). Thus, it may be beneficial to evaluate extrusion techniques to minimize the loss of tannins in supplements designed for ruminant diets.

A negative correlation does not necessarily indicate a direct cause–effect relationship; however, the negative association found between some bacterial taxa and the content of dietary fat, unsaturated fatty acids or tannins may indicate high sensitivity to these dietary components. For example, the negative correlation between the abundance BS11 and dietary fat may be due to toxic effects of fat on members of this bacterial family (van Lingen *et al.* 2017). Likewise, high sensitivity to tannins has been reported for *Prevotella* belonging to Paraprevotellaceae (Li *et al.* 2015).

Interestingly, when feeding extruded flaxseed to dairy cattle, the content of α-linolenic acid in blood serum and milk tended to increase (Kennelly 1996; Oeffner *et al.* 2013), and when cows consumed treatments containing the flaxseed-based products utilized in this experiment there was an increase in the concentration of omega-3 and total polyunsaturated fatty acids in omasal digesta and in milk (Moats *et al.* 2015). Bacterial species were not evaluated in this study; thus, we are unsure whether biohydrogenating micro-organisms were negatively affected. The family Christensenellaceae, which decreased with flaxseed inclusion, has been recently associated with low body mass index and reduced adiposity gain in non-ruminants (Goodrich *et al.* 2014). When cows consumed the extruded flaxseed-based product, body weight was not affected (Moats *et al.* 2015). Further investigation elucidating the activity and role of members of this bacterial family in the rumen and how they may impact production performance or fatty tissue accretion in dairy cattle is warranted.

In this experiment, the microbial profile of diets fed was not determined. Reports have shown that bacteria found in the diet could potentially affect ruminal micro-organisms (Ghorbani *et al.* 2002; Lettat *et al.* 2010), others have reported that the survival of some of these bacteria in the rumen is variable (Jeyanathan *et al.* 2016). More recently, Philippeau *et al.* (2017) reported that

direct-fed microbials did not affect ruminal micro-organisms or volatile fatty acid production. Therefore, it was not possible to determine associations between micro-organisms found in the diets, if any, and changes in the rumen microbial profile.

Overall, findings from this study indicate that flaxseed-based products tested were effective for replacing barley-based concentrate in lactating dairy rations without negative effects on predominant rumen bacterial taxa. However, the content of unsaturated fatty acids and tannins in the diets were negatively associated with some bacterial taxa found in lower proportions in the rumen such as Clostridiaceae, BS11, Paraprevotellaceae and Christensenellaceae; nonetheless, production performance and ruminal nutrient digestion were unaffected. The use of high-throughput DNA sequencing contributes to unravel the impact of diet composition on ruminal micro-organisms, strengthening our knowledge not only on dietary intervention methods to mitigate fatty acid biohydrogenation and improve milk quality, but also to prevent negative consequences on the ruminal bacterial population, feed digestion and rumen function.

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Conflict of Interest

Samodha C. Fernando, author of this publication has disclosed a significant financial interest in NuGUT LLC. In accordance with its Conflict of Interest policy, the University of Nebraska-Lincoln's Conflict of Interest in Research Committee has determined that this must be disclosed. The rest of the authors have nothing to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Beta diversity for bacterial communities in ruminal digesta samples for treatments CONT, a normal diet including barley silage, alfalfa hay and a barley-based concentrate with no flaxseed or faba beans; FLAX, inclusion of 11·4% of a nonextruded flaxseed-based product containing flaxseed, field peas and alfalfa; EXT, similar to FLAX, but the product was extruded; EXTT, similar to FLAX, but product was extruded and field peas were replaced by high-tannin faba beans.

Table S1 Effect of partially replacing a barley-based concentrate with different flaxseed-based products on bacterial richness estimates and diversity index for ruminal digesta samples from lactating Holstein dairy cows.