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Rumen Microbial Ecology And Rumen-Derived Fatty Acids: Determinants Of And Relationship To Dairy Cow Production Performance

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RUMEN MICROBIAL ECOLOGY AND RUMEN- DERIVED FATTY ACIDS: DETERMINANTS OF AND RELATIONSHIP TO DAIRY COW PERFORMANCE.

A Dissertation Presented

by

Laura M. Cersosimo

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Specializing in Animal, Nutrition, and Food Sciences

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Abstract

Rumen microbiota enable dairy cattle to breakdown fiber into useable energy for milk production. Rumen bacteria, protozoa, and fungi ferment feedstuffs into volatile fatty acids (VFA), the main energy source, while methanogens utilize fermentation by-products to produce methane. Milk fat contains several bioactive rumen-derived fatty acids (FA), including odd-chain FA (OCFA) and branched-chain FA (BCFA), important for maintenance of human health. The overarching dissertation goal was to determine which factors affect rumen methanogen and protozoal community structures and their metabolism products, while defining relationships between rumen microbiota and animal performance. Results presented contribute to the goals of providing new knowledge to dairy farmers, maintaining ruminant health, and enhancing bioactive FA in milk.

The first objective used next-generation sequencing techniques to determine if lactation stage and dairy breed affect rumen methanogen and protozoal community structures and protozoa cell FA compositions in Jersey, Holstein, and Holstein-Jersey crossbred cows at 3, 93, 183, and 273 days in milk (DIM). A core methanogen community persisted by lactation stage and breed. At 3 DIM, methanogen 16S rRNA gene sequences formed distinct clusters apart from 93, 183, and 273 DIM, reflective of the dietary transition period post-partum. The starch-utilizing protozoal genus *Entodinium*, was more abundant in Holsteins than in Jerseys and Holstein-Jersey crossbred cows and positively correlated with milk yield. Jerseys had greater iso-BCFA contents in protozoa and milk and protozoa of the genus *Metadinium*.

The second objective was to determine if supplementation of mixed cool-season grasses with annual forages (AF) alters the forage, microbial, and milk FA contents during typical periods of decreased pasture growth in Northeastern US. In short-term grazing (21d) of AF, ruminal VFA and major rumen-derived FA were not altered in bacterial and protozoal cells, suggesting little alteration of biohydrogenation and maintenance of ruminant health. In spring, milk contents of iso-15:0 and 17:0 per serving of whole milk were greater in control (CON)-fed cows, while contents of 12:0 and 14:0 per serving were greater in AF-fed cows. Contents of de novo FA and OCFA per serving of whole milk were greater in summer AF-fed cows than CON-fed cows, while total contents and BCFA did not differ, suggesting post-ruminal FA modifications.

The third objective was to characterize and relate the rumen microbiota from CON- and AF-fed cows to animal performance. Rumen protozoal taxa were not altered, while less abundant bacterial taxa (< 5%) were different in both periods. In spring, AF-fed cows had greater abundances of the methanogen species *Methanobrevibacter millerae*, whereas CON-fed cows had greater abundances of *Methanobrevibacter ruminantium*, potentially as a result of substrate availability. In summer, the protozoal genus *Diplodinium* was positively correlated with milk fat yield and %.

In conclusion, the work presented identifies several factors that influence rumen microbiota, rumen microbial FA, and milk FA, while providing new information to dairy farmers, researchers, and consumers.
Citations

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Chapter 1: Comprehensive Literature Review
1.1 Rumen Ecology

Dairy cattle rely on a consortium of rumen microbiota from all three domains of life (Archaea, Bacteria and Eukaryota) that co-occur to convert fiber to energy for milk production and produce methane. Metabolic functions (e.g., cellulose degradation) of several rumen microbial species are redundant relative to one another (Weimer, 2015), yet some microbial species are more abundant than others. Bacterial taxa such as those belonging to the genus *Prevotella* are able to perform a wide-range of functions, such as degradation of starch and fiber, while archaea (i.e., methanogens) belonging to the genus *Methanobrevibacter* utilize hydrogen and carbon dioxide or formate for methanogenesis (Weimer, 2015). Although “keystone species” (i.e., those greatly influencing rumen ecology and necessary for normal ruminal function) have not been clearly identified, rumen bacteria, protozoa, and methanogens have different functional roles that enable them to co-exist within the rumen environment (Mills et al., 1993) (Figure 1-1).

1.1.1 Rumen Development and Establishment of Microbiota

The rumen of a newborn calf is sterile and non-functional (Baldwin et al., 2004). During the first three days of life, the calf consumes colostrum, which is digested in the abomasum (i.e., glandular stomach) and the small intestine where nutrient absorption occurs. Because a calf has an active reticular groove located between the reticulum and rumen, and low starch intake during its first one to three weeks of life, milk flows to the abomasum instead of the rumen (Van Soest, 1994; Castro et al., 2016). Milk or milk replacer consumption will continue until the calf is weaned between three to four weeks of age (Baldwin et al., 2004). When calves transition from liquid to solid feed (e.g., hay,
grass, and grain), the reticular groove becomes non-functional, and solid feed flows into the rumen where microbial fermentation of feedstuff results in the production of volatile fatty acids (VFA). VFA are the main energy source for a ruminant and stimulate the proliferation of rumen papillae (Baldwin et al., 2004). The rumen grows in capacity from 30 to 70% of the total gastrointestinal tract and rumen microbial diversity measured as species richness and evenness increases (Warner et al., 1956; Castro et al., 2016). Cellulolytic bacteria and fungi (i.e., fermenters) and archaea (i.e., methanogens) are the first microbiota to establish in the rumen (Quigley, Schwab and Hylton 1985; Morvan et al. 1996). In a study by Guzman et al. [2015], rumen microbial colonization in Holstein bull calves occurred 20 minutes after birth and was characterized by the presence of methanogens, cellulolytic bacteria, and bacteria belonging to the genus Geobacter. It was not known, however, if these microbiota were transferred before or after calving (Guzman et al., 2015). Meale et al. [2016] suggested that the rumen and the gastrointestinal tract are inoculated with microbiota derived from feces, saliva, skin, and the vaginal canal of the dam at birth. Rumen ciliated protozoa were suggested to appear in the rumen at 21d of age and are the last microbiota to establish in the rumen, with the starch-utilizing protozoal genus, Entodinium, appearing first (Fonty et al., 1988).

1.1.2 Rumen Environment

The rumen is comprised of three layers, the gas (top), fibrous mat (middle), and liquid plus fine particles (bottom). The gas layer contains carbon dioxide (65.5%), methane (26.8%), nitrogen (7%), oxygen (0.5%), and hydrogen (0.2%) (Van Soest, 1994; Russell, 2002). The fiber mat acts as a first-stage separator (Sutherland, 1988) by retaining fine fiber particles that increase digestion time (Weidner and Grant, 1994).
Greater particle length and forage content make a thicker mat, whereas fine particles and greater contents of concentrate decrease mat thickness (Zebeli et al., 2007). The rumen fluid portion contains fine particles, rumen microorganisms, and nutrients, such as VFA and proteins (Van Soest, 1994).

VFA are short-chain fatty acids (FA) produced in the rumen by bacterial, protozoal, and fungal fermentation of organic matter (Dijkstra, 1994). VFA contribute to several metabolic processes that yield ATP (e.g., Kreb’s Cycle) therefore providing 70% of the energy required by the lactating dairy cow. The majority of ruminal VFA include acetate (two-carbon FA chain, 2:0), propionate (3:0), and butyrate (4:0). Proportions of acetate, propionate, and butyrate from mid-lactation dairy cows consuming a typical total mixed ration (TMR) are between 63-68%, 18-21%, and 11-15%, respectively (Schären et al., 2016). Ruminal acetate is the major energy source for the cow and is the main carbon source for milk fat synthesis. Cellulolytic bacteria, Butyrivibrio spp., Fibrobacter spp., and Ruminococcus spp., and proteolytic protozoa Entodinium caudatum and Eudiplodnium medium produce acetate (Castillo-Gonzalez et al., 2014). Butyrate is produced by the bacteria Butyrivibrio fibrisolvens and Megasphaera elsdenii and is converted to ketones during epithelial absorption across the ruminal wall (Castillo-Gonzalez et al., 2014). Propionate is the major substrate for gluconeogenesis in the liver (Dijkstra, 1994). The majority (90%) of the glucose supplied to the cow is provided by gluconeogenesis, with 50-60% derived from propionate (Reynolds et al., 1988). In addition to producing acetate, amylolytica bacteria Selenomonas spp. and Succinomonas spp. produce propionate (Castillo-Gonzalez et al., 2014). Isobutyrate and isovalerate are less abundant branched-chain VFA derived from deamination of valine and leucine,
respectively (Popova et al., 2011) and are necessary for the digestion of structural carbohydrates (e.g., cellulose, hemicellulose, and pectin) and for microbial protein synthesis (Liu et al., 2009).

Normal rumen pH in a lactating dairy cow is between 5.8-6.4 and varies by diet (Kolver and de Veth, 2002). High-concentrate diets, characterized by greater amounts of soluble carbohydrates (e.g., sugars) and starches, lower ruminal pH, whereas high-forage diets, characterized by neutral detergent fiber (e.g., cellulose, hemicellulose, and lignin), increase ruminal pH (Hook et al., 2011b). Sodium bicarbonate, a weak base occurring in the saliva or supplemented in the diet, acts to maintain normal ruminal pH by neutralizing excess hydrogen protons and increasing water intake (Mao et al., 2016). During the transition period from the dry period to early lactation, maintenance of normal ruminal pH is a challenge as diets formulated for lactating dairy cows contain greater amounts of concentrate (high energy) to offset a negative energy balance. Several factors contribute to a cow developing a negative energy balance, such as underdeveloped rumen papillae and increased VFA concentrations (Morgante et al., 2007; Dieho et al., 2016a; b). Underdeveloped papillae have a lower surface area for VFA absorption than fully developed papillae (Morgante et al., 2007). As VFA accumulate in the rumen, ruminal pH decreases and develops into subacute ruminal acidosis when ruminal pH is less than 5.6 for 3-5h (AlZahal et al., 2007).

1.1.3 Bacteria
Bacteria, are the most abundant rumen microorganisms (10^{10}-10^{11} cells/ mL rumen fluid) (Wright and Klieve, 2011), consisting of two core phyla, Bacteroidetes and Firmicutes (Weimer, 2015). Bacteria belonging to the phylum Bacteroidetes are
associated with plant starch breakdown, and those belonging to the phylum Firmicutes are associated with the breakdown of structural carbohydrates (Fernando et al., 2010).

Bacterial taxa *Butyrivibrio, Prevotella, Ruminococcus*, unclassified Ruminococcaceae, unclassified Bacteroidales, unclassified Clostridiales, and unclassified Lachnospiraceae were suggested to comprise the core rumen bacterial microbiome. A core microbiome is a population of microorganisms that remain stable regardless of diet and host genetics (Weimer, 2015). These seven bacterial taxa were 67% of the total bacterial sequences in a data set of 742 rumen fluid samples collected from 32 ruminants from 35 countries (Henderson et al., 2015b). Bacteria belonging to the genera *Ruminococcus* and *Butyrivibrio* are the most abundant members of the phylum Firmicutes, while bacteria belonging to the genus *Prevotella* are the most abundant within the phylum Bacteroidetes (Stevenson and Weimer, 2007; Weimer, 2015).

Rumen bacteria have several metabolic functions that enable them to alter or breakdown feed components. Both *Ruminococcus* and *Butyrivibrio* belong to the order Clostridiales and the family Lachnospiraceae. Both genera have cellulolytic capabilities and are more abundant in forage-fed than concentrate-fed ruminants (Henderson et al., 2015). The bacterial species *Butyrivibrio fibrosolvens* is involved in ruminal biohydrogenation of polyunsaturated fatty acids (PUFA) to stearic acid (18:0, SA) (Maia et al., 2010). Some strains of *Butyrivibrio fibrosolvens* that contain greater contents of linoleate isomerase (an enzyme that converts PUFA to conjugated linoleic acids, CLA) are more tolerant to linoleic acid (18:2 c9,c12, LA) than others (Fukuda et al., 2006) and *Butyrivibrio proteoclasticus* P18, which is predominantly found in fish oil, can
biohydrogenate docosahexaenoic acid (22:6 c4, c7, c10, c13, c16, c19, DHA), whereas the former can not (Jeyanathan et al., 2016).

Bacteria belonging to the species *Ruminococcus albus* contain cellulosomes that enable it to adhere to and digest cellulose and its genome encodes cellulases and hemicellulases (Suen et al., 2011). Species belonging to the genus *Prevotella* are characterized by their proteolytic capabilities. *Prevotella ruminicola* contains dipeptidyl peptidase activity that enables it to break peptide bonds (Wallace et al., 1997). In contrast, several cultured strains have broad abilities to hydrolyze starch and ferment sugars and amino acids (Weimer, 2015) that may enable them to have distinct niches within the rumen (Avguštin et al., 1997). Lastly, it was suggested that unclassified taxa belonging to the Ruminococcaceae, Lachnospiraceae, and Clostridiales are highly abundant and competitive in ruminal bacteria, and play a key role in forage digestion (Kim et al., 2011).

1.1.4 Protozoa

Little is known about the role rumen protozoa play in digesting feedstuff in comparison to rumen bacteria. Because of the increased popularity of studying prokaryotes for methane mitigation strategies and feed efficiency and the challenges of maintaining them in culture, there are more outdated rumen protozoal studies than current (within the last five years). Entodiniomorphida and Vestibuliferida are two orders of protozoa, characterized by different structures and metabolic activities (Williams and Coleman, 1992). Protozoa belonging to the order Entodiniomorphida have cilia around their oral cavity and on different areas of their body, whereas protozoa belonging to the order Vestibuliferida have cilia around their entire body which enable them to attach to
plant cell surfaces (Russell, 2002). Protozoal genera belonging to the order Entodiniomorphida include *Diplodinium*, *Epidinium*, *Entodinium*, *Eudiplodinium*, *Metadinium*, *Ostracodinium*, and *Ophyroscolex*, while the order Vestibuliferida is less diverse and consists of the genera *Dasytricha* and *Isotricha* (Wright et al., 1997; Wright, 2009).

Over half a century ago, culture-dependent strategies categorized rumen protozoa into different types, O, A, and B (Eadie 1962). Type O protozoal populations include *Entodinium* spp., *Dasytricha*, and *Isotricha*; type A consist of type O ciliates plus *Polyplastion* spp., *Diploplastron*, and *Ophryoscolex* and type B contain type O ciliates, *Epidinium* spp., *Eudiplodium maggi*, and *Eremoplastron bovis* (Eadie 1962). In contrast, when culture-independent techniques (*i.e.*, Roche 454-pyrosequencing) and four-primer pairs targeted the protozoal 18S rRNA gene in moose, the identified protozoal populations were comprised of a mixture of all three protozoal types (Ishaq and Wright, 2014). Similar results were obtained when the rumen protozoal population was characterized by the Illumina MiSeq sequencing platform from dairy cattle offered five different diets (Tapio et al., 2016). Newbold et al. [2015] suggested in a review of rumen protozoa that the current methods used to target the protozoal 18S rRNA gene might be unreliable at measuring α-diversity (*i.e.*, relative abundances of genera within a sample), but reliable at measuring β-diversity (*i.e.*, relative abundances of genera between samples). This is because larger protozoal species, such as *Epidinium caudatum* contain more 18S rRNA gene copies in their genomes than smaller protozoal species, such as *Entodinium caudatum* (Dehority, 1993). Currently, no studies have quantified the 18S rRNA gene copies per cell (Newbold et al., 2015). Therefore, it is important to
acknowledge these limitations when interpreting results from culture-independent protozoal diversity studies.

Protozoa, especially those from the order Entodiniomorphida, engulf starch granules that are broken down by amylase into maltose units. Starch, a non-fiber carbohydrate, is a major component of high-concentrate diets, and is typically fed to dairy cows transitioning from a non-lactating to lactating physiological state (Hook et al., 2011a). Franzolin and Dehority [1996] observed greater protozoal densities in steers fed a high-concentrate diet than those fed a high-forage diet, which was suggested to be associated with rate of saliva production and rate of passage of ruminal particulate matter. Hook et al. [2011] increased content of concentrate in the diet of dairy cows, but a type A protozoal population persisted. Mendoza et al. [1993] observed in sheep that rumen protozoa reduced the rate of starch digestion and digestibility in the rumen and shifted the starch digestion to the small intestine. As a result, it was hypothesized that the protozoa stabilized the ruminal pH of sheep and preventing the host from developing subacute ruminal acidosis (Mendoza et al., 1993). Prior to this study, a positive correlation between rumen protozoal density and feed efficiency was observed, indicating that a higher protozoal density, shifted starch digestion to the small intestine while increasing feed efficiency (Mendoza et al., 1993).

Protozoa belonging to the orders Entodiniomorphida and Vestibuliferida were suggested to degrade fiber, but from different approaches (Orpin, 1984). Protozoa belonging to the genus *Isotricha* attach to the plant cell surface, whereas *Epidinium caudatum* and *Eudiplodinium maggi* use their oral cavities to attach to plant fibers (Orpin, 1984). When defaunated (no protozoa), monofaunated (one protozoal species),
and faunated sheep consumed four different diets (starch, sucrose, lactose, and inulin), the presence of rumen protozoa resulted in a 3-10% increase in lignocellulose digestibility (Jouany and Senaud, 1979). Although monofaunated sheep with Polyplastron spp., did not have improved fiber digestibility, those monofaunated sheep with Entodinium spp. had improved feed digestibility (Jouany and Senaud, 1979).

Rumen protozoa are involved in the degradation of both dietary and microbial proteins. Feed-derived true protein and non-protein N are broken down to amino acids or ammonia. Members of the order Entodiniomorphida release high concentrations of protease to degrade insoluble protein, but are incapable of metabolizing soluble proteins (e.g., casein). Alternatively, protozoa belonging to the order Vestibuliferida comprise of multiple proteases that enable them to metabolize insoluble and soluble proteins (Jouany, 1996). Collectively, rumen protozoa are capable of modifying the supply of protein from feed-derived and bacterial protein (through predation). Greater rumen ammonia concentrations are typically observed in faunated ruminants versus defaunated, indicating that rumen protozoa do play a role in the breakdown of dietary protein (Jouany, 1996). As ammonia concentrations increased, so does the quantity of nitrogen in the urine, thus, decreasing the ruminant’s utilization of nitrogen towards milk production.

1.1.5 Archaea


Methanogens are strict anaerobes, with fastidious growth requirements. They require different substrates for methane production, and exist synergistically with bacteria
and symbiotically with rumen ciliate protozoa. Presently, there are 120 species of methanogens, representing 33 different genera. The differences in morphology and utilization of different substrates by different methanogens enable them to be found in a diverse number of habitats. Knowledge of this ecosystem is rapidly accumulating, particularly with the advent of molecular biology and culture-independent technologies.

The domain Archaea is comprised of four phyla: Crenarchaeota, Korarchaeota, Nanoarchaeota, and Euryarchaeota. The Euryarchaeota, the largest phylum, includes the methanogenic archaea, which produce methane, the halophilic archaea, that live and grow in hypersaline, and some extremely thermophilic archaea, that thrive at relatively high temperatures between 45 and 122 °C. The methanogenic archaea, or methanogens, are the most frequently studied and observed group of Archaea within the rumen (Paul et al., 2012).

Methanogens represent less than 1% (10^7-10^9 cells/mL rumen fluid) of the total rumen microbial population, and maintain a synergistic relationship with bacteria and a symbiotic relationship with protozoa (Wright & Klieve, 2011). Additionally, some methanogens are associated with the ciliated protozoa. Specifically, an adhesion protein identified in Methanobrevibacter (Mbr) ruminantium M1 enables this species to bind to hydrogen-producing protozoa (e.g., Entodinium) (Ng et al., 2016). Protozoa support methanogenesis by transferring hydrogen to the methanogens, while methanogens utilize hydrogen that would normally cause an inhibitory effect on protozoal metabolism. Methanogens mainly utilize hydrogen to reduce carbon dioxide to methane.

The production of methane is a normal product of rumen fermentation that acts as a pathway for the deposition of metabolic hydrogen produced (Kumar et al., 2011).
Methane generated in the rumen is a significant electron sink that generates an electrochemical gradient across the cell membrane to produce ATP (Klieve, et al., 2012; Stewart & Bryant, 1988). Since hydrogen is utilized by the methanogens to produce methane, the concentration of hydrogen is maintained at low levels in the rumen. Hydrogen is typically derived as a catabolic product from both bacteria and protozoa where it is utilized by methanogens to reduce carbon dioxide to methane. Most methanogens belonging to the following genera are capable of using hydrogen to reduce carbon dioxide to make methane: *Methanobrevibacter, Methanobacterium*, *Methanothermobacter, Methanothermus*, and some members of the genus *Methanosarcina* (Ferry and Kastead, 2007).

Species belonging to the genus *Methanobrevibacter* are the predominant methanogens in the rumen (Henderson et al., 2015b; Tapio et al., 2016). Species of *Methanobrevibacter* include: *Mbr. smithii, Mbr. gottschalkii, Mbr. thaueri, Mbr. ruminantium, Mbr. olleyae, Mbr. millerae, Mbr. wolinii, Mbr. woesi* and *Mbr. arboriphilus*. It has been observed that *Methanobrevibacter*-related sequences were distributed between two major taxonomic clades, the *smithii-gottschalkii-millerae-thaueri* (SGMT) clade and the *ruminantium-olleyae* (RO) clade (King et al., 2011; St-Pierre and Wright, 2012). Although hydrogen and carbon dioxide are the main substrates for methanogenesis by members from both phylogenetic clades, the species strain *Mbr. ruminantium M1* lacks the isoenzyme methyl coenzyme M reductase II (McrII), expressed at high ruminal concentrations of hydrogen, but contains the enzyme, methyl coenzyme M reductase I (McrI), which is expressed in low concentrations of hydrogen
(Leahy et al., 2010). Conversely, *Mbr. olleyae* strain DSM16632 and *Mbr. millerae* strain DSM16643 have both isoenzymes (McCabe et al., 2015).

Methanogens are also able to use other substrates to meet growth requirements and produce methane. An estimated 22.1% of rumen methanogens were able to grow with hydrogen and methyl groups, whereas 77.7% of methanogens were hydrogenotrophic (Henderson et al., 2015b). Methanogens, such as *Methanosphaera stadtmanae* and the Methanomassiliicoccales isolate ISO4-H5 use methanol and methylamines derived from pectin degradation (e.g., beet and citrus pulp) (Li et al., 2016). In the rumen, cellulolytic bacteria, fungi, and ciliate protozoa release formate (HCOO-) into the rumen fluid where it is utilized by methanogens (Ellis et al., 1990). The majority of formate is converted to hydrogen and carbon dioxide via formate hydrogenases and then used in the production of methane by methanogens (Hungate et al., 1970). Methanogen genera *Methanosarcina* and *Methanosaeta* are rarely identified in the rumen (< 0.015% relative abundance) as they utilize acetate and have low growth rates (Henderson et al., 2015b).

### 1.2 Microbial Cell Membrane Fatty Acids

As the consumption of individual FA in milk contributes to the maintenance or improvement of human health, there is great interest in understanding the relationship between rumen-derived microbial cell and milk FA compositions. Microbial cells contain low amounts of intracytoplasmic lipids (< 1%), while the cell membrane is the principal site for lipid accumulation (Ratledge and Wilkinson, 1988). Branched-chain FA (BCFA) are saturated with methyl groups on the penultimate (*iso*) or antepenultimate (*anteiso*) carbon atom unique to bacteria and other microorganisms. Both branched- and odd-chain
FA (OBCFA) have been identified in bacterial and protozoal cell membranes. OBCFA contents of rumen methanogen cell membranes, however, have not been described in the literature (Vlaeminck et al., 2005; Jenkins et al., 2015).

With respect to human health, iso-15:0 treatment inhibited growth of prostate carcinomas and hepatocarcinomas (Yang et al., 2000) and caused apoptosis of breast cancer cell lines (Wongtangtintharn et al., 2005). Serum contents of iso-BCFA were greater in non-obese versus obese women, suggesting the potential health benefits of an iso-BCFA-rich diet (Mika et al., 2016). 15:0 and 17:0 are primarily derived from dairy products and the most prevalent odd-chain FA (OCFA) found in microorganisms and cows’ milk (Wang et al., 2011). Total OCFA in blood plasma were greater from cognitively healthy individuals than from those with Alzheimer’s disease (Fonteh et al., 2014).

Rumen microbial cells contain several preformed FA (> 18 carbons) that are diet- and rumen-derived. Dietary polyunsaturated FA (PUFA) include the essential FA, LA and α-linolenic acid (ALA, 18:3 c9,c12,c15), which are both precursors of signaling molecules (Fonteh et al., 2014). Dietary supplementation of vaccenic acid (VA, 18:1 t11), a typical derivative of ruminal biohydrogenation of ALA and LA, decreased adipocyte size by 7% in rats (Mohankumar et al., 2012) and improved in vitro insulin secretion in human islets (Wang et al., 2016).

1.2.1 Bacterial Cell Membrane Fatty Acids
Dietary PUFA are bacteriostatic and exist only at low concentrations within bacterial cells (Maia et al., 2010). Bacteria, such as those belonging to the genus Butyrivibrio, overcome this issue through biohydrogenation of unsaturated FA (UFA) to
During biohydrogenation, CLA isomers and several *cis*- and *trans*-18:1 isomers, including VA, are formed as intermediates (Figure 1-2). Rumen bacteria are divided into two groups, where A and B have different contributions to rumen biohydrogenation.

Group A bacteria (*e.g.*, *Butyrvibrio* spp. and *Ruminococcus albus*) form VA, while Group B (*e.g.*, *Fusocillus*) form SA (Kemp and Lander, 1984). Once bacterial cells leave the rumen and flow to the duodenum, they may undergo chain-elongation (*e.g.*, 15:0 to 17:0) or *de novo* FA synthesis (<16 carbons) in the mammary gland, and subsequently contribute to the milk FA contents (Vlaeminck et al., 2015).

Ruminal bacteria synthesize OBCFA for incorporation into their cell membranes (Kaneda, 1991). OCFA and *iso*-BCFA give bacteria a biological advantage with melting points 1-2°C lower than straight-chain FA with n-1 atoms (*e.g.*, 16:0) that increase cell membrane fluidity (Or-Rashid et al., 2007). Microbial OCFA are constructed via chain elongation of propionate (3:0) or valerate (5:0) (Kaneda, 1991). Ruminal isobutyrate and isovalerate are BCFA precursors, originating from diet-derived branched-chain amino acids, valine, isoleucine, and leucine (Liu et al., 2009). However, the majority of OBCFA in bacterial cells are thought to originate from bacterial FA synthase activity, instead of precursor availability (Vlaeminck et al., 2006a). Variability in the bacterial cell OBCFA contents may reflect changes in the bacterial populations. For example, bacterial *iso*-FA are predominantly found in cellulolytic bacterial populations (*e.g.*, *Ruminococcus flavefaciens*), whereas bacterial antit*iso*-15:0 are greater in sugar- and pectin-fermenting bacteria (*e.g.*, *Prevotella ruminicola*) (Vlaeminck et al., 2006a; Bessa et al., 2009).

In comparison to research in rumen protozoal cells, several studies have identified factors that alter rumen bacterial OBCFA contents. The amount of dietary
forage was linked to a greater rumen bacterial OBCFA profile from 14 dry cows and four mid-lactation Holstein cows, which was likely a result of a change in the bacterial population (Bas et al., 2003; Vlaeminck et al., 2006b). Two studies observed that liquid-associated (17.0% and 17.3%, respectively) bacteria have greater contents of OBCFA than solid-associated bacteria (5.9% and 7.3%, respectively) (Vlaeminck et al., 2006b; Bessa et al., 2009). Because bacterial FA are associated with the bacterial cell wall, Gram-positive bacteria (e.g., *Streptococcus bovis*, *Ruminococcus* spp.) have lower total FA concentrations than Gram-negative bacteria (e.g., *Prevotella*). Yet, BCFA contents have been associated with Gram-positive bacteria (Kaneda, 1991; Vlaeminck et al., 2006b).

### 1.2.2 Protozoal Cell Membrane Fatty Acids

Protozoal cells engulf and store chloroplasts, whereas rumen bacteria do not (Huws et al., 2009). Chloroplasts contain the majority of plant ALA within their thylakoid membranes (Hall et al., 1974). During mastication of forage, some plant cells are lysed and FA are released from the thylakoid membranes (Martin et al., 1994). It was determined that more than 50% of plant cells were found intact post-mastication (Martin et al., 1994). When Hereford-Friesian steers were provided perennial ryegrass (PRG), protozoa belonging to the genus *Epidinium* were saturated with chloroplasts (Huws et al., 2009). In another study where Holstein x Friesian steers consumed PRG, 5% of the protozoa identified (*i.e.*, *Diplodonium* and *Polyplastron*) were saturated with chloroplasts, whereas protozoa from steers consuming a straw-based diet with low levels of chloroplasts were not (Huws et al., 2012). Notably, *Epidinium* spp. accumulated PRG chloroplasts in their cytoplasms, whereas Hall et al. [1974] observed that *Entodinium* spp.
stored spinach chloroplasts in their food vacuoles. Chloroplast storage in food vacuoles results in the loss of chloroplast morphology, while storage in cytoplasm is suggested to protect the chloroplasts from microbial digestive enzymes. Because of disparities exist in the location of chloroplast storage, greater dietary intake of total n-3 FA by one group of cows over another does not necessarily indicate that there will be greater total n-3 FA contents in the rumen and thus protozoal cells.

Rumen protozoa contain greater concentrations of CLA and VA than rumen bacteria (Harfoot, 1978; Lorenço et al., 2010), CLA, however, was undetectable in *Isotricha protostoma, Entodinium furca, and Entodinium nanellum* from sheep monofaunated with these protozoal species (Devillard et al., 2006). The mechanism in which CLA and VA accumulate in protozoa is unknown. Rumen protozoa do not biohydrogenate UFA, nor do they desaturate SA to VA (Devillard et al., 2006). Co-localization of intra-protozoal chloroplasts and bacteria within food vacuoles and biohydrogenation by bacteria were suggested as potential mechanisms of CLA and VA accumulation. Protozoa, such as *Entodinium caudatum*, had 31% of their cellular volume composed of bacteria (Williams and Coleman, 1992), whereas total protozoal contamination by bacteria was 18% (Huws et al., 2012). Therefore, it is possible that this protozoal species and others obtain CLA and VA through bacterial predation (Williams and Coleman, 1992).

A greater flow of ruminal ALA to the duodenum was hypothesized to be achieved by increasing the chloroplast content of the diet, this, however, was a challenge as protozoal 18S rRNA concentrations were low, indicating rumen retention (Huws et al., 2012). By increasing the duodenal flow of protozoa with greater proportions of bioactive
FA, such as LA, ALA, and OBCFA, it is thought that this could enhance the bioactive FA profile of milk, however several studies identified 16:0 and SA to comprise the majority of protozoal cell FA contents from four dry Holstein cows and three Holstein dairy cows, respectively (Emmanuel, 1974; Or-Rashid et al., 2007; Sultana et al., 2011) (Table 1-1). Therefore, increasing the duodenal flow of protozoa may also increase the flow of these SFA, less desired by the milk consumer (Huws et al., 2009).

1.3 Key Determinants of Rumen Microbial Community Structures

1.3.1 Diet

Dietary components, such as starch, protein, fiber, and lipids affect the rumen microbial community structures of lactating dairy cattle. Between 2011-2016, several studies identified the less abundant rumen microbial populations from dairy cows provided different diets by using next-generation sequencing (NGS) and other strategies (e.g., clone libraries) (Table 1-2). These dietary studies, however, are more limited in comparison to those studies that have focused on rumen bacteria. Because rumen bacteria represent greater than 90% of the rumen microbial population, emphasis has focused on characterizing their community structures instead of those of methanogens, protozoa, or fungi that exist at much lower abundances within the rumen. Nevertheless, the less abundant microbiota, specifically protozoa and methanogens, have unique functions in the rumen that are important to the rumen environment and ruminant nutrition.

1.3.2 Breed

Because dairy breeds differ in animal performance (e.g., milk and fat yields) (White et al., 2001), there is interest in what role host genetics may play in influencing
their rumen microbial communities. Previously, ruminal 16S rRNA gene clones belonging to the methanogen species *Methanobrevibacter millerae* were more frequently identified in Jersey than Holstein cows, while *Methanosphaera* spp. were more frequently found in Holsteins (King et al., 2011). Although limitations of this study included the use of clone libraries (365 total sequences), pooled samples from nine Holsteins and 10 Jerseys, this was the first study to compare rumen methanogen communities between dairy cattle breeds. Densities of the rumen bacterial species *Ruminococcus albus* identified by quantitative PCR were greater in lactating Holstein and Holstein-Jersey crossbreed than in Jersey cows, however, abundances of the species *Fibrobacter succinogenes*, rumen bacterial and protozoal densities did not differ by breed (Beecher et al., 2014). Abundances of operational taxonomic units (OTU) belonging to the bacterial family Lachnospiraceae were greater in multiparous Holstein (n = 5) than in Jersey (n = 4) dairy cows and principal coordinate analyses revealed clustering of 16S rRNA gene sequences by breed, suggesting that the rumen bacterial community differs between these two breeds (Paz et al., 2016). Another conclusion from this study was that future studies that evaluate rumen microbial communities must increase animal numbers to increase statistical power (Paz et al., 2016).

When rumen microbiota from Nili Ravi and Murrah buffaloes (*i.e.*, the most common dairy breeds in Asia) were compared, rumen bacterial and protozoal populations identified via Roche 454-pyrosequencing techniques, did not differ by breed, while ruminal acetate and propionate were greater in Murrah buffaloes (Lin et al., 2015). Notably, relative abundance of the *Methanobrevibacter ruminantium* clade was greater in Nili Ravi (12%) than Murrah (7%) buffaloes. Although this study was the first to
compare the rumen microbiota from two commonly used buffalo breeds, the significance of this study was limited by low numbers of animals (3 per breed) and sequences (i.e., 2,085 18S rRNA gene sequences) (Lin et al., 2015).

1.3.3 Lactation Status
Previous research has provided little knowledge about the community structure of rumen bacteria and no information about the rumen methanogen and protozoal taxa across a lactation period. Instead, the transition stage (three weeks before calving to three weeks post-calving) and the relationship with the rumen microbiota has been the focus of several studies, rather than a full lactation period (approximately 305d). This is because cows may develop health issues (e.g., ruminal acidosis and ketosis) that disrupt normal ruminal functions and subsequent animal performance occurring when energy and glucose supplies are not sufficient and body fat mobilization is increased (Wang et al., 2012). At the prepartum period ruminal densities of the species *Prevotella ruminicola* were lower than at 1 and 7d postpartum (Wang et al., 2012). Furthermore, the rumen Bacteroidetes to Firmicutes ratio of 10 Holstein cows doubled 1-3d post-calving (12:1) in comparison to 3d before calving (6:1), this ratio however, remained stable four and eight weeks post-calving (Pitta et al., 2014a). Bacterial Shannon diversity indices and relative abundances of the protozoal class Litostomatea generated from Illumina MiSeq sequencing analyses were greater in prepartum than postpartum cows (Lima et al., 2014). The relative abundances of Bacteroidetes and Firmicutes did not differ at early (76-82 days in milk (DIM)), mid- (151-157 DIM), or late-lactation (251-257 DIM) (Jewell et al., 2015).
1.4 Relationship of Rumen Microbiota to Animal Performance

Rumen bacterial community structures were thought to influence dairy cow physiological parameters and therefore their production traits or performance (Jami et al., 2014). Yet, little research has attempted to delineate this relationship with rumen protozoa and methanogens. Feed efficiency, milk production, and milk protein and fat yields are important measurements of dairy cattle performance. Feed efficiency is calculated as residual feed intake (RFI, \(i.e.,\) the difference between actual feed intake and expected requirements for maintenance) in both beef and dairy cattle or as the gross feed efficiency (\(i.e.,\) energy-corrected milk (ECM)/DMI) in dairy cows. If a better understanding of this relationship is defined through research, then the rumen microbial populations may be manipulated to potentially enhance dairy cattle performance.

1.4.1 Rumen Bacteria and Animal Performance

The rumen bacterial community was suggested to shape the physiological parameters of the host, including milk production and solids (\(e.g.,\) fat, lactose and protein) from dairy cows (Jami et al., 2014). Previously, the identification of different rumen bacterial groups between efficient and inefficient feedlot steers indicated that specific bacteria may be associated with feed efficiency (Guan et al., 2008), however, no significant correlations were observed between the rumen bacterial community and RFI in lactating dairy cows (Jami et al., 2014). Different indications from these studies may have been as a result of different techniques to analyze bacterial diversity (denaturing gel gradient electrophoresis versus 454-pyrosequencing) and host species (Guan et al., 2008; Jami et al., 2014).
Several studies have focused on the relationship between rumen bacterial taxa and milk fat yield. Bacterial species such as *Streptococcus bovis* (lactate-producer) and *Megasphaera elsdenii* and *Selenomonas ruminantium* (lactate-utilizers) are associated with subacute ruminal acidosis (rumen pH < 5.6). This can result in decreased milk and milk fat production and a greater proportion of lactate-producers to lactate-utilizers (Hook et al., 2011a). Abundances of the lactate-producing bacterial species *Streptococcus bovis* and *Lactobacillus* spp. were shown to increase during the transition period as the rumen pH dropped (Wang et al., 2012). Abundances of bacteria belonging to the species *Prevotella bryantii* and *Fibrobacter succinogens* decreased, whereas *Streptococcus bovis* increased from cows induced with milk fat depression (Rico et al., 2015). A linear relationship was found between the ratio of Firmicutes to Bacteroidetes and milk-fat yield (Jami et al., 2014), while another study did not observe this relationship (Lima et al., 2014). Discrepancies between the two studies occurred as the former study consisted of 15 Holstein cows and one time point, while the latter study consisted of 48 primiparous and 67 multiparous Holstein dairy cows observed at two time points.

### 1.4.2 Protozoa and Animal Performance

Currently, there is a gap in knowledge regarding the relationship of the less abundant rumen microbiota, archaea and protozoa, to animal performance. Instead the focus has been on the relationship between the total numbers of rumen archaea or protozoa, not individual taxa in relationship to animal performance. Total rumen protozoal counts from cows with diet-induced milk fat depression were linearly associated with milk fat % and milk fat concentrations of 18:1 trans-10. This study, however, did not identify rumen protozoa that might be associated with decreased milk
fat (Rico et al., 2015). Furthermore, it was reported that a type A protozoal population 
(Polyplastron, Entodinium, Isotricha, and Dasytricha) persisted in non-lactating dairy 
cows before and after they were induced with subacute ruminal acidosis, yet the two time 
points were not statistically compared (Hook et al., 2011b). Numbers of rumen protozoa 
and microscopic counts of the less abundant protozoal genus Dasytricha were positively 
associated with milk fat % from four different farms, however each farm only contained 
three to five cows (Shimado et al., 1989). Milk fat yields and total protozoal numbers 
were greater from Holstein cows supplemented with palm oil than those without 
supplementation (Kirovski et al., 2015). The authors hypothesized that the protozoa had 
an extended exposure to energy precursors (e.g., amino acids, glycerol, and lactate) when 
supplemented with palm oil.

1.4.3 Archaea and Animal Performance

As methane emissions are associated with decreased feed efficiency (Johnson 
and Johnson, 1995), researchers have been interested in identifying this relationship, 
while no known study identified the relationship between rumen methanogen taxa and 
milk production parameters. Total methanogen numbers and abundances of individual 
methanogen species such as Methanobrevibacter smithii, Methanobrevibacter 
ruminantium, and Methanosphaera stadtmaneae did not correlate to DMI of high forage 
or low forage diets in beef cattle (Carberry et al., 2014). Conversely, 16S rRNA gene 
clones with 94% identity to Methanobrevibacter ruminantium were associated with DMI 
in steers (Zhou et al., 2010), while Methanosphaera stadtmaneae were more prevalent in 
inefficient steers than efficient steers (Zhou et al., 2009).
1.5 Conclusions

The introduction of NGS (e.g., Roche 454-pyrosequencing, Illumina MiSeq) has enabled the field of rumen microbiology to expand and to identify microbial taxa that are not easily identified via culture-dependent techniques. With culture-independent techniques, numerous studies have focused on targeting the rumen bacterial 16S rRNA gene with respect to ruminant health and dairy cattle performance (Jami et al., 2014; Pitta et al., 2014). However, few studies have used NGS techniques to identify rumen methanogens and protozoa from lactating dairy cows. Instead, studies solely focus on rumen bacteria, use other methods such as denaturing gel gradient electrophoresis (Zhou et al., 2010), clones libraries (King et al., 2011), or report total archaeal and protozoal densities (number per mL rumen fluid). As rumen microbiota interact with one another and form symbiotic relationships in the rumen, it is important that we identify and quantify the relative abundances of the less abundant microbial taxa. This will enable us to gain a better understanding of which factors influence the rumen microbial community structures and to better define the relationship between rumen microbiota and dairy cattle performance.

Rumen-derived FA in milk are important to the maintenance of human health. Several studies identified the rumen bacterial and protozoal cell FA profiles, however there are several unanswered questions. Though it is known that the bacterial and protozoal FA profiles differ from one another and that diets with different chloroplast levels alter the UFA profile of their cells, previous studies used less than five animals, steers (Huws et al., 2009, 2012), sheep (Devillard et al., 2006), or dry cows (Or-Rashid et al., 2007). As the long-term goal is to enhance the rumen-derived FA in milk, it is
important that more studies determine the rumen microbial FA profile from lactating
dairy cows and determine which factors (e.g., breed, lactation stage, or feeding strategies)
influence it so that future studies can measure and a potential increase the flow of the
duodenum and mammary gland.

1.6 Overarching Dissertation Hypothesis and Objectives

Hypothesis

Based on indications from previous studies, it was hypothesized that breed, lactation stage, and alternative feeding strategies are key factors affecting the rumen methanogen and protozoal community structures and their metabolites.

Main Objective

Determine if breed, lactation stage, and alternative feeding strategies affect rumen methanogen and protozoal community structures and rumen protozoal cell FA profiles with emphasis on rumen-derived FA.

Specific objectives of each experiment listed below were based on information from previous studies and conclusions of my dissertation literature review.

Experiment 1:

An observational study using molecular biology and NGS techniques was conducted at the University of Vermont Paul Miller Research Farm from May 2013 to May 2014 with 22 primiparous dairy cattle (7 Holstein, 7 Holstein-Jersey Cross, 8 Jersey). The specific objective was to test the effects of dairy breed and lactation stage on the rumen methanogen and protozoal communities and their metabolites.
Experiments 2 and 3:

Once effects of breed and lactation stage on rumen microbiota were identified, two separate, feeding trials using different annual forage mixtures (Spring: barley, wheat, rye, triticale, and hairy vetch; Summer: oat, buckwheat, and chickling vetch) were performed with mid-lactation Jersey cows under organic dairy management practices at the University of New Hampshire in May and July 2015. Specific objectives were to:

1) compare rumen microbial communities, microbial cell (i.e., bacterial and protozoal) and milk FA contents between cows provided cool-season grasses and cows provided cool-season grasses plus annual forages.

2) define relationships between rumen microbiota and animal performance.

By accomplishing these objectives, my research contributes to new information in regards to the determinants of rumen microbial community structures, the relationship between rumen protozoal taxa and animal performance, and the microbial and milk FA profiles.
1.7 References


Yang, Z., S. Liu, and X. Chen. 2000. Induction of apoptotic cell death and *in vivo* growth inhibition of human cancer cells by a saturated branched-chain fatty acid, 13-


Table 1-1 Identification of rumen protozoal cell fatty acid profiles from previous publications

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<td>8.0</td>
<td>-</td>
<td>7.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OCFA</td>
<td>3.7</td>
<td>-</td>
<td>3.7</td>
<td>3.7</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>18:1 t11</td>
<td>-</td>
<td>7.5-8.0</td>
<td>6.6</td>
<td>5.5</td>
<td>6.0</td>
<td>13.4</td>
</tr>
<tr>
<td>18:2 c9,c12</td>
<td>17.0</td>
<td>15-20</td>
<td>7.2</td>
<td>8.8</td>
<td>5.5</td>
<td>1.3</td>
</tr>
<tr>
<td>18:3 c9,c12,c15</td>
<td>1.9</td>
<td>1.0</td>
<td>1.5</td>
<td>0.5</td>
<td>0.5</td>
<td>2.8</td>
</tr>
<tr>
<td>18:2 c9,t11</td>
<td>-</td>
<td>1-3</td>
<td>1.3</td>
<td>1.6</td>
<td>0.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

1 S:C, straw to concentrate, 2 PRG, perennial ryegrass
Table 1-2 Summary of publications from 2011-2016 demonstrating the effects of diet on rumen methanogen and protozoal community structures from dairy cows.

<table>
<thead>
<tr>
<th>Title</th>
<th>Reference</th>
<th>Diet</th>
<th>Methodology</th>
<th>Major Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Microbiome analysis of dairy cows fed pasture or total mixed ration diets”</td>
<td>de Menezes et al. 2011</td>
<td>TMR Pasture (plant species and contents not identified)</td>
<td>Terminal restriction fragment length polymorphism (T-RFLP)</td>
<td>Methanogen communities varied by diet, while protozoal communities were characterized by pronounced inter-animal variations.</td>
</tr>
<tr>
<td>“Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows”</td>
<td>Hook et al. 2011</td>
<td>Chopped hay (69 g/kg dry matter, DM) vs. high grain (409 g/kg DM)</td>
<td>Methanogens-clone libraries Prototiza-microscopy</td>
<td>Archaeal Shannon diversity indices were lowest at 3 weeks (high-concentrate diet). Type A protozoal populations were observed at 0 (baseline), 3, and 6 weeks.</td>
</tr>
<tr>
<td>“Effect of dietary protein concentration and coconut supplementation on nitrogen utilization and production in dairy cows”</td>
<td>Lee et al. 2011</td>
<td>Deficient in metabolizable protein (MP) with coconut oil added to TMR</td>
<td>Methanogens-denaturing gradient gel electrophoresis (DGGE) Prototiza-microscopy</td>
<td>Total protozoal and Entodinium sp. counts were decreased, while methanogen populations were not affected.</td>
</tr>
<tr>
<td>“Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive”</td>
<td>Zhou et al. 2011</td>
<td>TMR plus fibrolytic enzymes (endoglucanase and xylanase)</td>
<td>DGGE</td>
<td>Total methanogen densities did not differ between cows supplemented with or without enzymes. Addition of enzymes altered the methanogen DGGE profile.</td>
</tr>
<tr>
<td>“Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation”</td>
<td>Belanche et al. 2012</td>
<td>High and low fiber or protein TMR</td>
<td>Real-time PCR and Microscopy</td>
<td>The low protein TMR decreased protozoal and methanogen densities, while fiber diets increased their densities.</td>
</tr>
<tr>
<td>“Supplementation of increasing amounts of linseed oil to dairy cows fed total mixed rations: Effect on digestion, ruminal fermentation characteristics, protozoal populations, and milk fatty acid”</td>
<td>Benchaar et al. 2012</td>
<td>TMR with and without linseed oil (2, 3, and 4% inclusion)</td>
<td>Microscopy</td>
<td>Counts of protozoal genera Entodinium, Epidinium, and Isotricha spp. did not differ by diet.</td>
</tr>
<tr>
<td>Study Title</td>
<td>Authors</td>
<td>Diet/Condition</td>
<td>Method/Technique</td>
<td>Result/Findings</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td>------------------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>“Methanogenic population and CH₄ production in Swedish dairy cows fed different levels of forage”</td>
<td>Danielsson et al. 2012</td>
<td>TMR (50:50 or 90:10 forage to concentrate)</td>
<td>T-RFLP</td>
<td>Total methanogens and methanogens related to the order Methanobacteriales were greater on the 50:50 diet.</td>
</tr>
<tr>
<td>“Biodiversity and composition of methanogenic populations in the rumen of cows fed alfalfa hay or triticale straw”</td>
<td>Kong et al. 2013</td>
<td>Alfalfa hay or triticale straw</td>
<td>Quantitative fluorescence in situ hybridization</td>
<td>Relative abundances of methanogen species varied between cows, but not by diet.</td>
</tr>
<tr>
<td>“Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range”</td>
<td>Henderson et al. 2015</td>
<td>Worldwide forage, browse, or concentrate-based diets from 742 samples</td>
<td>454 GS FLX</td>
<td>Diets with high pectin contents had greater abundances of <em>Methanosphaera</em> sp., <em>Entodinium</em> and <em>Ophyroscolex</em>, whereas <em>Eudiplodinium</em> was more abundant with low levels of starch and pectin.</td>
</tr>
<tr>
<td>“Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows”</td>
<td>Kumar et al. 2015</td>
<td>High forage (pre-calving, 80%) to high grain (post-calving, 50%)</td>
<td>454 GS FLX</td>
<td>Shannon diversity of methanogens and relative abundances of <em>Methanobrevibacter</em> and <em>Methanosphaera</em> did not differ by diet.</td>
</tr>
<tr>
<td>“Rumen protozoal communities are dynamic over a dietary switch from conserved forage to pasture”</td>
<td>Bainbridge et al. 2016 (abstract)</td>
<td>Conserved forage vs. diverse pasture (15 species)</td>
<td>Illumina MiSeq</td>
<td>Protozoal community structures were dynamic during the transition from conserved forage to pasture (e.g., <em>Entodinium</em> 2-79% abundance)</td>
</tr>
<tr>
<td>“Diet-induced changes of redox potential underlie compositional shifts in the rumen archaeal community”</td>
<td>Friedman et al. 2016</td>
<td>High-grain (65% grain) Low-grain (0% grain)</td>
<td>Illumina MiSeq</td>
<td>Cows fed the non-grain diet had diet-unique OTU (orders Methanosarcinales and Methanomicrobiales), absent in grain-fed cows.</td>
</tr>
<tr>
<td>“Feeding ground flaxseed (GFX) to lactating dairy cows decreases the ruminal proportion of Archaea, but does not change the major species of”</td>
<td>Soder et al. 2016 (abstract)</td>
<td>TMR, 0%, 5%, 10%, and 15% GFX supplementation</td>
<td>Illumina MiSeq</td>
<td>Relative abundances of <em>Methanobrevibacter</em> and <em>Methanosphaera</em> spp. responded linearly to GFX treatment.</td>
</tr>
<tr>
<td>Study Title</td>
<td>Authors</td>
<td>Experimental Design</td>
<td>Method</td>
<td>Results</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>“Oral samples as non-invasive proxies for assessing the composition of the rumen microbial community”</td>
<td>Tapio et al. 2016</td>
<td>Grass silage with 5 experimental diets (no additional fat, 50 g/kg DM myristic acid (MA), rapeseed oil, safflower oil, or linseed oil)</td>
<td>Illumina MiSeq</td>
<td>Inclusion of MA decreased proportions of methanogens relative to bacteria, changed rumen fungal community without altering the methanogen and protozoal communities.</td>
</tr>
</tbody>
</table>
| “Ruminal methanogen community in dairy cows fed agricultural residues of corn stover, rapeseed, and cottonseed meal”                          | Wang et al. 2016 | 1. alfalfa and corn silage  
2. residue mixture plus soybean  
3. residue mixture                                                                                                                                  | Clone libraries  | Principal component analyses showed that methanogen communities clustered differently by diet.  
Methanogen diversity increased when agricultural residues were fed. |
Figure 1-1 Key nutrients fermented by rumen bacterial and protozoal and substrates utilized by methanogen species. Information from Pond et al., 2005; St-Pierre et al., 2015; Li et al., 2016b were used to depict metabolic functions of rumen microbiota.
Figure 1-2 Synthesis of ruminal biohydrogenation intermediates and end-products from dietary linoleic and linoleic acids. Figure illustrations are based on the metabolic pathways described in a review by Shingfield and Wallace, 2014.
Chapter 2: Breed And Lactation Stage Alter The Rumen Protozoal Fatty Acid Profiles And Community Structures In Primiparous Dairy Cattle.

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Notes

The authors declare no competing financial interest.

Keywords: bioactive fatty acids, conjugated linoleic acids, ciliates, Holstein, Jersey, Holstein-Jersey crossbreeds
2.1 Abstract
The protozoal FA composition and community structure are important to dairy
cattle nutrition and their products. The purpose of the study was to observe if the rumen
protozoal fatty acid (FA) profiles and protozoal community structure differed by breed
and lactation stage. At 93, 183, and 273 days in milk (DIM), whole rumen digesta
samples were collected from 7 co-housed Holstein (H), 8 Jersey (J), and 7 Holstein-
Jersey crossbreed (C) cows. Rumen protozoal linoleic acid was higher at 183 (8.08%) and
273 (8.30%) DIM than at 93 (5.68%)DIM. Oleic acid was the most abundant
protozoal unsaturated FA (10.12%). Protozoal rumenic acid (RA), and protozoa of the
genus *Metadinium* were higher in J (9.93%) than in H (0.52%) and C (0.96%). Protozoa
belonging to the genus *Entodinium* were more abundant in H (45.16%) than in J
(23.37%) and C (30.16%). In conclusion, breed and DIM affected several protozoal FA
and genera.

2.2 Introduction
The rumen is an anaerobic environment, containing microorganisms (bacteria,
fungi, and protozoa) that ferment feedstuff into volatile fatty acids (VFA), the main
energy source for animal production. Bacteria make up the majority of the rumen
microbiome (10^{10}-10^{11} cells/mL rumen digesta)^1. The protozoal density (10^{4}-10^{6} cells/mL
rumen digesta)^1 is much lower, yet, they account for 40-50% of the microbial biomass^2.
Rumen protozoa are anaerobic ciliates representing a diverse group of over 30
characterized genera^3. The two major groups include the orders Entodiniomorphida and
Vestibuliferida. Entodiniomorphids (*e.g.*, *Entodinium, Eudiplodinium, Polyplastron,
*Metadinium spp.*) have cilia around their cytostomes, feed on plant surfaces, and engulf
starch granules. In contrast, Vestibuliferids (e.g., *Dasytricha* and *Isotricha* spp.), have cilia covering their entire body\(^4\)–\(^6\). Compared to rumen bacteria, much less is known about rumen protozoa. Like rumen bacteria, protozoa breakdown starch, fiber, and proteins\(^6\)–\(^2\).

Polyunsaturated fatty acids (PUFA) inhibit the growth of rumen bacteria (e.g., *Butyrivibrio fibrisolvens*) as a result of their double bonds, and thus, rumen bacteria biohydrogenate feed-derived PUFA to saturated fatty acids (SFA)\(^7\). Rumen protozoa engulf chloroplasts\(^8\) that are high in the PUFA linoleic acid (LA; 18:2 \(\text{9c,12c}\)) and \(\alpha\)-linolenic acid (ALA; 18:3 \(\text{9c,12c,15c}\)). Although several studies demonstrated that protozoa do not biohydrogenate unsaturated fatty acids (UFA)\(^8\)–\(^10\), Or-Rashid *et al.*\(^11\) suggested that protozoa might convert stearic acid (SA; 18:0) to vaccenic acid (VA; 18:1 \(\text{11t}\)) through \(\Delta\text{11}\)-desaturase activity. It has been suggested that biohydrogenation of intra/protozoal chloroplasts may occur as protozoa engulf bacteria into their vacuoles\(^12\).

The lipid membranes of protozoa contain more UFA, including biohydrogenation intermediates, such as VA and conjugated linoleic acids (CLA), than bacteria\(^11\). Since rumen protozoa provide UFA for duodenal absorption that are subsequently incorporated into meat and milk\(^11\), they have the potential to contribute to a more appealing product to the consumer.

In the United States, Holstein and Jersey cows are the most popular breeds of dairy cattle. Holsteins are known for their high milk production, whereas Jerseys are known for their milk solids\(^13\). Holstein-Jersey crossbreeds are of interest because they combine favorable qualities from both breeds. Previous research using clone libraries, suggested differences in the rumen methanogen communities between lactating Holstein
and Jersey dairy cows\textsuperscript{14}, whereas the abundance of the rumen bacterium, \textit{Ruminococcus albus} was higher in Jersey cows\textsuperscript{15}. Although Beecher \textit{et al.}\textsuperscript{15} observed no differences in the density of rumen protozoa in Holstein, Jersey, and Holstein-Jersey crossbreed cows, protozoal taxa were not identified.

Few studies have identified rumen protozoal genera\textsuperscript{16–19}, while no studies have identified the rumen protozoal fatty-acid (FA) profile at different DIM and/or in three breeds of dairy cattle. To date, the majority of rumen protozoal research has focused on culture-dependent strategies, rather than culture-independent metagenomic strategies, such as next-generation sequencing (NGS) of the eukaryote-specific 18S rRNA gene. Furthermore, Lima \textit{et al.}\textsuperscript{17}, used a NGS platform to demonstrate differences in protozoal taxonomic classes before and after calving when cows transition from a high-neutral detergent fiber (NDF) to a high-starch diet, but, did not identify 18S rRNA gene sequences to species or genus levels at different DIM. Little is known about how DIM and breed affect rumen protozoa. As the rumen protozoal community contributes to the fermentation of carbohydrates, and the protozoal FA composition contributes to the milk FA composition, it is important to gain knowledge about each component.

We hypothesized that breed and DIM would alter the rumen protozoal FA composition, community structure, and density in co-housed, primiparous Holstein, Jersey, and Holstein-Jersey crossbreeds consuming a total mixed ration at 93 (early lactation), 183 (mid-lactation), and 273 (late-lactation) DIM. Our objectives were to (1) identify and quantify the protozoal FA compositions, (2) classify the rumen protozoal 18S rRNA gene sequences to genera, (3) measure the protozoal cell densities, and (4)
determine if correlations exist between rumen protozoal genera and protozoal FA compositions at 93, 183, and 273 DIM.

2.3 Materials and Methods

2.3.1 Experimental Design

A total of 22 primiparous dairy cattle (7 Holstein (H), 8 Jersey (J), and 7 Holstein-Jersey crossbreeds (C)) were co-housed in free stalls at the University of Vermont’s Paul Miller Research Complex in South Burlington, VT from May 2013-May 2014. All cows calved within a two-month period. The University of Vermont’s Institutional Animal Care and Use Committee approved all animal sampling procedures under protocol #13-031.

2.3.2 Diet

At the start of their lactation, cows were transitioned to a TMR diet of corn silage (52.3% as-fed), haylage (15.9%), and concentrate (31.8%) that was consumed throughout the study. The concentrate contained corn grain (24.6%), citrus pulp (19.1%), amino max (16.4%), soybean meal (16.4%), canola meal (10.9%), amino enhancer (5.5%), calcium carbonate (2.5%), sodium sesquinate (2.2%), salt (1.2%), magnesium oxide (0.7%), trace mineral premix and vitamins (0.4%), zinc methionine (0.1%), and rumensin (<0.1%). Each week, for three consecutive days, TMR samples were collected during AM milking before cows had access to it. Since the cows were co-housed in a free stall, 22 individual TMR samples from each cow were not collected. The three samples were composited weekly and then by time point. Cumberland Valley Analytical Services (Hagerstown, MD) analyzed the TMR samples and determined the nutrient compositions (Table 2-1). The TMR diet was intended to have low variability by DIM, however the author’s note
the numerical increase in crude protein (17.0%) at 273 DIM. Uncontrollable limitations to the nutrient composition may have included the effect of season, inconsistent on-farm mixing of the TMR, and the storage of the corn silage and haylage. TMR FA analyses as described by Bainbridge et al.\textsuperscript{20} were measured at 93, 183, and 273 DIM (Table 2-2). In order to compare the diet FA profile to the protozoal FA profile, each FA is shown as a percentage of the total FA (%) for a given time point.

2.3.3 Fractionation of Whole Rumen Digesta

Prior to esophageal intubation, cows were removed from feed for 3h and whole rumen digesta samples were collected at 0900 h. A total of 500 mL of whole rumen digesta was collected via stomach tubing (1.27 cm wide, 200 cm length milk hose) at 93, 183, and 273 DIM. Rumen pH was immediately measured (Accumet Portable Laboratory pH meter, model AP110, Fisher Scientific) and a 50 mL aliquot was taken for VFA analyses. All rumen pH and VFA molar concentrations from the present study were previously reported\textsuperscript{21}.

The protocols described by Or-Rashid \textit{et al.}\textsuperscript{11} and Lee \textit{et al.}\textsuperscript{22} were used to fractionate the whole rumen digesta samples, but the following adaptations were made. Each sample was diluted by 30% with MB9 Buffer (2.8 g NaCl/L, 0.1 g CaCl\textsubscript{2}·2H\textsubscript{2}O/L, 0.1 g MgSO\textsubscript{4}·7H\textsubscript{2}O/L, 2.0 g KH\textsubscript{2}PO\textsubscript{4}/L, and 6.0 g Na\textsubscript{2}HPO\textsubscript{4}/L) and 1.5 mL of 1% methyl cellulose was added. The samples were blended in a Bella rocket blender (Sensio Inc., Montréal, CN) for 30s and let stand at 4°C for 1h. Only final protozoal fractions containing <5% plant matter were used for FA analyses.
2.3.4 Protozoal lipid extraction and FA analyses

Protozoal fractions (15-20 mL) were lyophilized for 48h in a freeze-dryer (Labconco, Kansas City, MO). Freeze-dried protozoal samples (230-280 mg) were weighed into 20 x 150 mm test tubes with Teflon-lined screw caps. Protozoal FA lipid extractions and transesterification was based on the method of Vlaeminck et al. 23. Protozoal fatty acid methyl esters (FAME) were identified using gas chromatography (GC) with mass spectrometry (GC/MS) in electron ionization mode. Samples were run on a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) equipped with a split/splitless injector (1:40 split ratio) using a Rtx-2330 (90% bis cyanopropyl/10% phenyl cyanopropyl polysiloxane; 105 m x 0.25 mm x 0.1 µm; Restex, Bellefonte, PA) column. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The temperatures of the injector, ion source, and interface were 260°C, 200°C and 240°C, respectively. The GC oven program was as follows: initial temperature of 45°C, held for 4 min, programmed at 13°C/min to 150°C, held for 27 min, then programmed at 3°C/min to 215°C, held for 35 min. GC/MS analyses were performed in full scan mode (m/z 45-500). The injection volume was 1 µL of the FAME mixture. Integration and quantification were performed with GCMS Solutions software (ver. 2.72; Shimadzu, Kyoto, Japan). FAME were identified by comparison with known standards (Nu-Check Prep 463 and 674 (NuCheck Prep, Inc., Elysian, MN), Supelco 37 component FAME mixture (Sigma-Aldrich, Saint Louis, MO), individual iso and anteiso branched-chain fatty acids from 10 to 20 carbons (Larodan Fine Chemicals AB, Malmo, Sweden)) and comparison of spectra averaged over the width of the GC peak, with background subtraction, to the National Institute of Standards
and Technology mass spectral library. These were based on a similarity factor of at least 95% between the unknown and the library spectra.

**2.3.5 Microbial DNA extraction and PCR amplification of the 18S rRNA gene**

Microbial DNA was extracted from whole rumen digesta samples using the methods established by Yu and Morrison and the adaptations defined by Cersosimo et al. The rumen protozoal-specific primer pair, P-SSU-316F (5’-GCTTTCGWTGCTAGTATT-3’) and GIC758R (5’-CAACTGTCTCTATKAYCG-3’) was used to amplify the V3-V4 hypervariable regions and signature regions 1-2 of the 18S rRNA gene on a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA). Each 50 µL PCR reaction contained, 2 µL undiluted DNA extract, 31.5 µL ddH2O, 2.5 µL P-SSU-316F primer, 2.5 µL GIC758R primer, and 1 µL (dNTP), 10 µL HF buffer (Thermo Scientific, Waltham, MA), and 0.5 µL Thermo Scientific Phusion DNA polymerase. Protozoal amplicons were generated under the following conditions: hot start (94°C, 240s), followed by 35 cycles of denaturation (94°C, 30s), annealing (55°C, 30s), and extension (72°C, 60s). A final extension of 72°C for 6m was performed in the last cycle. Molecular Research DNA Laboratories (Shallowater, TX) sequenced the purified protozoal PCR products (25 µL) with the Illumina MiSeq v.3 platform.

**2.3.6 Bioinformatics Analyses of 18S rRNA gene sequences**

Access to the raw 18S rRNA gene sequence data set is available through the National Center for Biotechnology Information’s Sequence Read Archive, under the study accession number [SRP064980].
All bioinformatics analyses were performed with the bioinformatics tool, MOTHUR (v. 1.33.3)\textsuperscript{29}. The 18S rRNA gene sequences were quality checked with an in-house Perl script to a Phred score of 30 or above. The command, trim.seqs was used to remove barcodes and create a file that contained which sequences belonged to which sample. The command, unique.seqs determined how many unique sequences were in the data set. Align.seqs performed a Needleman-Wunsch pairwise alignment of the unique sequences and used the BLAST protozoal reference file made by Ishaq and Wright\textsuperscript{27}. Further adjustments to the alignment were made by manually scanning each sequence for any misalignments. Once the sequences were aligned, chimera.uchime was used to identify chimeric sequences\textsuperscript{30}. Any chimeric sequences were removed with an accnos file of chimera sequence names and the command, remove.seqs.

The 18S rRNA gene sequences were classified into taxa by using a taxonomy file of known rumen protozoa\textsuperscript{27} in conjunction with the classify.seqs command using the method=wang parameter. The nearest-neighbor distance method was used to determine the genetic distance between sequences at a cutoff of 4\%. The phylip file generated from dist.seqs was used to cluster the sequences into operational taxonomic units (OTU). The OTUs were classified into nearest related taxa with classify.otu. The OTU-based diversities, Shannon diversity index, Good’s coverage, and Inverse Simpson index were calculated by summary.single.

2.3.7 Real-time PCR
A protozoal standard was generated from whole rumen digesta from a dry Holstein cow housed at the Paul Miller Research Farm (South Burlington, VT). Protozoal cells were microscopically counted and diluted to 8.0 x 10\textsuperscript{0}-8.0 x 10\textsuperscript{4} cells/mL. Protozoal
DNA was extracted using the previously mentioned microbial DNA extraction method. All real-time PCR reactions were completed with the primer pair (P-SSU-316f, P-SSU-539R) and thermal cycler protocol established by Sylvester et al.\textsuperscript{26}. The master mix for each 25µL reaction was: 12.5 µL SYBR green, 2.5 µL of each primer, and 6.5 µL ddH\textsubscript{2}O. One µL of the unknowns, standards, positive and negative controls were run in triplicate on a 96-well plate.

### 2.3.8 Statistical Analyses

Data were statistically evaluated as repeated measures with the PROC MIXED model in SAS (v.9.4, SAS Inst. Inc., Cary, NC). The model included cow as a random effect and breed, DIM, and the interaction of breed by DIM as fixed effects. All Pearson correlations (r) were generated with PROC CORR and a heatmap was made with the online graphing and analytical tool, Plotly. Differences between breed, DIM, and breed x DIM were declared significant at $P<0.05$.

### 2.4 Results

#### 2.4.1 Diet FA Composition

Throughout the lactation, LA was the most abundant FA (41.1%) identified in the TMR, followed by oleic acid (OA) (20.8%), palmitic acid (PA) (16.7%), and ALA (12.0%). PUFA comprised 52.8% of the total protozoal FA content. At 183 DIM the sum of the n-3 FA (10.0%) was numerically lower than at 93 DIM (14.4%), while n-6 FA were numerically higher at 183 DIM (42.7%) and lower at 93 DIM (39.3%) (Table 2-2).

#### 2.4.2 Protozoal FA Composition

Over the lactation period, SFA were the most abundant group of rumen protozoal FA with stearic acid (SA) (30.5%) and PA (29.1%) being the most predominant
FA identified, respectively. LA and ALA were the only PUFA detected, with a greater abundance of LA (7.3% and 1.2%, respectively). Branched-chain FA (BCFA) were less than 6.0% of total FA, while 18:1 *trans* isomers ranged between 14.7% and 20.2% of total FA (Table 2-3).

Breed and DIM affected the protozoal FA compositions. RA was more abundant in protozoa from J (1.8%) than H (1.0%) and C (1.1%) (*P* = 0.01). The BCFA, protozoal *iso*-16 was more abundant in J (1.3%, *P* = 0.02) than in H (1.0%)(*P* =0.02). Breed did not affect the total protozoal percentages of BCFA, SFA, 18:1 *trans* isomers, odd-chain FA, nor the most abundant FA, PA and SA (Table 2-3).

DIM had a greater effect than breed on the rumen protozoal FA profiles (Table 2-3). Notably, PA, SA, total BCFA were not affected by DIM, while the total *anteiso*-BCFA, SFA and 18:1 *trans* isomers were affected (Table 2-4). *iso*-17:0 was elevated at 273 DIM (0.34%, *P*<0.01) when compared to 93 (0.24%), and 183 (0.25%) DIM, respectively. OA was the most abundant UFA and higher at 183 DIM (11.9%, *P*<0.05) than at 93 (10.3%), and 273 (8.2%) DIM. LA was less abundant at 93 DIM (5.7%, *P*<0.001) than at 183 (8.1%) and 273 DIM (8.3%). RA was highest at 93 (1.7%) and 183 (1.4%) DIM than at 273 DIM (0.8%) (*P*<0.05 ) (Table 2-4).

**2.4.3 Rumen Protozoa Taxa, Density, and Diversity Measures**

A total of 1,518,385 18S rRNA gene sequences were generated. Sequence reads were between 253-510 base pairs (bp) with an average sequence length of 448 bp. The mean and standard deviation of the sequences per breed were: 20,286 ± 7,600 (H), 20,250 ± 12,035 (J), and 28,628 ± 9,281 (C). Of the total sequences, 165,704 (10.9%) were unique.
The majority of sequences (95.1%) belonged to rumen ciliates from the order Entodiniomorphida, while the remaining sequences (4.9%) belonged to the order Vestibuliferida. Only two genera, *Dasytricha* and *Isotricha*, were from ciliates belonging to the order Vestibuliferida. The most abundant protozoal genera (>10% 18S rRNA gene sequence abundance) identified were *Entodinium*, *Epidinium*, *Eudiplodinium*, and *Metadinium*, along with unclassified genera belonging to the family Ophyroscolecidae (Table 2-5). Low abundant genera included *Dasytricha*, *Diplodinium*, *Isotricha*, *Ophryoscolex*, *Ostracodinium*, and *Polyplastron* (Table 2-5).

DIM had a greater effect than breed on the protozoa genera identified (Table 2-5). Members from the genera *Entodinium* and *Metadinium* and were affected by breed ($P<0.05$). J had higher abundances of *Metadinium* spp. (9.9%) than C (1.0%), H (0.5%) ($P<0.01$). The ciliates belonging to the genera *Entodinium*, *Eudiplodinium*, *Ostracodinium*, *Polyplastron*, and unclassified Ophyroscolecidae were affected by DIM ($P<0.05$; Figure 2-1). At 93 DIM, *Entodinium* and *Polyplastron* spp. were more abundant, while *Epidinium* and *Eudiplodinium* spp. were less abundant (Figure 2-1A). The genus *Ostracodinium* was higher in abundance at 273 DIM (6.6%) than at 93 (0.9%) and 183 (0.2%) DIM ($P<0.001$) (Figure 2-1B).

Rumen protozoal densities (log cells/mL rumen digesta) and diversity measures did not vary by breed (H: 5.88, J: 5.80, and C: 5.77). Densities were higher at 183 DIM (6.5) and 273 DIM (6.4) than at 93 DIM (5.1) ($P<0.001$). The average number of OTU, Shannon diversity index, and Chao I richness estimator were higher at 273 DIM than at 93 and 183 DIM ($P<0.05$) (Table 2-6).
2.4.4 Correlations Between Protozoal Genera And Fatty Acid Composition

Few correlations were identified between protozoal genera and the protozoal FA composition. 18S rRNA gene sequences related to the genus *Metadinium* showed the strongest correlations to the protozoal FA composition (Figure 2-2). The strongest correlation was between *Metadinium* and RA ($r= 0.49$, $P<0.01$). The most abundant protozoal genus, *Entodinium* showed negative correlations ($r= -0.31$, $P<0.01$; $r= -0.49$, $P<0.001$) to LA and ALA, respectively, and a positive correlation to OA ($r=0.41$, $P<0.001$).

2.5 Discussion

2.5.1 Rumen Protozoal FA Compositions

In agreement with previous studies, PA and SA were the predominant FA identified in the rumen protozoal fractions$^{11,31}$. Unlike previous studies$^{11,31}$, PA percentages were lower relative to SA. In contrast, the PA content from these to previous studies was numerically higher at 41% and 38%, respectively, but less than four cows, at different stages of lactation, were used. Broad and Dawson$^{32}$ demonstrated that suspensions of *Entodinium caudatum*, ingest SA, the main product of rumen bacterial biohydrogenation. Since protozoa belonging to the genus *Entodinium* were the most abundant (30.3%) in the present study, a higher amount of SA could have been taken up by them, thus contributing to the total rumen protozoal FA composition. Furthermore, Williams$^{33}$ observed that protozoa incorporate PA from feed, indicating that the amount of PA in the diet influences the amount in rumen protozoal membrane FA. Lastly, there is still conflicting evidence$^{11,8}$ about the ability of rumen protozoa to biohydrogenate LA
and ALA from the diet, making it difficult to determine how and where exactly rumen protozoa obtain SA.

Findings relative to rumen protozoal LA and ALA from previous studies vary. When Hereford-Friesian steers were fed fresh perennial ryegrass, the percentage of protozoal LA (5%) was lower than ALA (10%), while no difference was observed on a hay-based diet (LA: 5%, ALA: 4%). This finding was attributed to the higher ALA content of the ryegrass diet and intracellular chloroplasts at 39.7% of the protozoa. Or-Rashid et al. found similar results in dry Holsteins with rumen protozoa containing 7.2% LA and 1.5% ALA of total FA. DIM, but not breed, affected the rumen protozoal LA and ALA content. LA was higher at 183 and 273 DIM and ALA was highest at 273 DIM. However, chloroplasts were not quantified, limiting the ability to determine how much chloroplasts contributed to the present findings.

Since CLA and OA are considered bioactive FA related to human health and are more prevalent in rumen protozoa than in bacteria, research has focused on their quantification. Rumen protozoal CLA percentages were highest at 93 and 183 DIM. Unlike OA, CLA are not FA found in plant material, but are intermediates of rumen bacterial biohydrogenation (e.g., Butyrivibrio fibrisolvens). Previous work suggested that the rumen protozoa obtain these FA from the ingestion of rumen bacteria and preferentially incorporate CLA into their membranes. Notably, some protozoa occurring in sheep, such as Isotricha prostoma, Entodinium nanellum, and Entodinium furca, do not contain CLA. Although protozoal CLA and the genus Metadinium were positively correlated, conclusions could not be drawn between them. This is partially because the 18S rRNA gene sequences were not long enough to confidently classify to species level,
and that there are only few reference sequences for the rumen ciliates. Future research could further determine the relationship between rumen protozoal species at different lactation stages by using monofaunated (one species of protozoa) cows or by sequencing the whole 18S rRNA gene.

Although Or-Rashid et al.\textsuperscript{11} used dry Holstein cattle, CLA, VA, and OA percentages were numerically similar (1.9\%, 6.6\%, and 7.8\%, respectively) compared to the present study (1.3\%, 3.9\%, and 10.1\%, respectively). Regardless of lactation state (i.e., dry versus lactating), OA was the most abundant UFA identified in rumen protozoa\textsuperscript{11}. Devillard \textit{et al.}\textsuperscript{8} also demonstrated this finding in rumen protozoa from adult male sheep. Interestingly, the highest OA content in both the diet and in rumen protozoa was observed at 183 DIM. Since some rumen protozoal species engulf chloroplasts\textsuperscript{34}, it is conceivable that they sequestered the OA from the diet.

In agreement with previous studies\textsuperscript{11,37}, the percentage of rumen protozoal BCFA and odd-chain FA relative to UFA were low. Rumen bacteria produce and incorporate BCFA and odd-chain FA to maintain cell membrane fluidity\textsuperscript{38}, yet, it is thought that protozoa maintain their cell membranes via UFA and not BCFA or odd-chain FA. Williams and Dinusson\textsuperscript{37} did not find detectable amounts of branched 14:0, 15:0, 16:0, or 17:0 from suspensions of various rumen protozoa. In contrast, when suspensions of rumen protozoa were washed with \textsuperscript{14}C-labeled isoleucine, it was suggested that a 2-methyl butyrate precursor, produced by isoleucine was used to produce BCFA, \textit{anteiso}-15 and \textit{anteiso}-17\textsuperscript{31}. Furthermore, Emmanuel\textsuperscript{31} showed that propionate was used for the production of 15:0 and 17:0 and that (2-\textsuperscript{14}C) PA was converted to 17:0 by rumen protozoa, which may explain why these FA were the most abundant odd-chain
FA found throughout the lactation in all three dairy breeds. Although past studies investigated protozoal lipid-biosynthetic properties, future work should expand upon past research to determine how much bacterial ingestion and carbon-chain elongation by rumen protozoa affects the BCFA and odd-chain FA profiles.

2.5.2 Rumen Protozoal Diversity And Density

Few studies have used NGS techniques\textsuperscript{17,19} to generate rumen protozoal 18S rRNA gene sequences in dairy cattle. The present study is the first to compare rumen protozoal communities between three dairy breeds and at different stages in lactation. Kittelmann \textit{et al.}\textsuperscript{19} used 454-pyrosequencing to identify rumen protozoal genera in two Holstein-Jersey crossbreeds, one beef Holstein cross, and in one Holstein cow, but the cows were on different diets (e.g., pasture vs. silage) and of different ages. Relative to the present study and regardless of diet in the Kittelmann \textit{et al.}\textsuperscript{19} study, the protozoal genera, \textit{Diploplastron, Entodinium,} and \textit{Ostracodinium} were present in all four cows. Similarly, low abundances of protozoa genera from \textit{Diploplastron} and \textit{Isotricha} were observed. In contrast, two out of the four cows had a greater abundance of \textit{Dasytricha} (>20%), but they consumed pasture-based diets. Lima \textit{et al.}\textsuperscript{17} used the Illumina MiSeq platform to characterize the rumen protozoa in Holsteins one week before and after calving, yet, classified sequences to taxonomic class, not genus. Greater than 90% of the protozoal 18S rRNA gene sequences belonged to the starch-utilizing class Litostomatea with a higher pre-calving relative abundance.

When dry Holstein dairy cattle were fed a high-concentrate diet (75%) and a control diet, the genera \textit{Entodinium, Dasytricha, Isotricha, Polyplastron}, and \textit{Ophyroscolex} were identified via microscopy, not NGS\textsuperscript{16}. Although the genera identified
were similar to those from the present study, their abundances were not determined. Vogels et al.\textsuperscript{18} used scanning electron microscopy and identified \textit{Entodinium simplex} and \textit{Ostracodinium obtusum} in 25 Holstein dairy cattle and \textit{Eudiplodinium maggii} in 80% of the cows. Karnati \textit{et al.}\textsuperscript{39} showed that the abundance of the genus \textit{Entodinium} was much higher in a dairy cow on a 50:50 forage:concentrate diet, while a cow on an alfalfa-based diet had more \textit{Dasytricha ruminantium}.

In agreement with previous studies\textsuperscript{18,19,40}, \textit{Entodinium} was the most prevalent rumen protozoa genus identified. Species belonging to the genus \textit{Entodinium} ingest bacteria and contain amylases that digest engulfed starch grains. H had a higher abundance of protozoa belonging to the genus, \textit{Entodinium} than J and C. To the authors’ knowledge, no study has compared the percent abundance of \textit{Entodinium spp.} between breeds, so further research (\textit{e.g.}, measurement of amylase activity) should be performed to determine why this difference was observed. Furthermore, the abundance of \textit{Entodinium} was highest at 93 DIM, roughly peak lactation, where DMI is highest in a typical lactation curve.

Few studies have measured rumen protozoal diversity parameters. Previous studies indicated that the microbial diversity increases with higher dietary fiber\textsuperscript{41,42}. Although the protozoal community was more diverse at 273 DIM, the lignin percentages were within 1% of each other, while the NDF percentages were within 2.5% and lowest at 273 DIM. Belanche \textit{et al.}\textsuperscript{41} also showed that the rumen microbial diversity decreased as the cow’s dietary N requirement was decreased from 110% to 80% in multiparous Holstein cows, but did measure the diversity in rumen protozoa. The crude protein
content at 273 DIM was 3.2% and 2.1% higher than at 93 and 183 DIM, respectively, but does not indicate that it caused an increase in the protozoal diversity.

2.5.3 Correlations Between Rumen Protozoa And Fatty Acid Compositions

Previously, the content of VA and CLA of several protozoal species was identified, however, no correlations were made with other FA. Few correlations were drawn between rumen protozoal taxa and rumen protozoal FA. Higher percentages of protozoal CLA and the genus *Metadinium* were observed in J cows, while a positive correlation was shown between these two observations (r=0.49, *P*<0.001). Although a positive correlation was present, future work could use cattle monofaunated with *Metadinium* spp. to quantify the rumen protozoal CLA. One limitation was that whole rumen digesta samples were used to identify the rumen protozoa present, whereas rumen protozoa from fractionation were used to quantify the fatty acid profiles. As a result of fractionation, the loss of some protozoal species may have occurred (*e.g.*, loss during cheesecloth filtration). Current work is comparing the rumen protozoa taxa from whole digesta versus fractionation.

In conclusion, the present study was an observational study that used current NGS technologies and FA analysis techniques to provide new information about rumen protozoal genera and FA compositions in lactating dairy cows. This study was a platform for future research, with the overall goals of gaining more knowledge about dairy cattle nutrition and enhancing the FA profile of the milk. The rumen protozoal FA composition and community structures were more affected by days in milk than dairy breed. Breed affected two rumen protozoal genera, *Entodinium* and *Metadinium*. Interestingly, RA and the protozoal genus, *Metadinium* were higher in Jersey cows and were positively
correlated to each other. Although protozoal LA and ALA were abundant, the amount of chloroplasts engulfed by the protozoa were not quantified. Therefore, future work is warranted to determine how rumen protozoa incorporate and utilize biohydrogenation intermediates and how much chloroplasts contribute to the protozoal FA composition.

2.6 Funding Sources
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2.7 Acknowledgements
The authors thank UVM farm staff, Matt Boudette, Scott Shumway, Doug Watkin for their assistance with the project animals. A special thanks to the University of Vermont undergraduate students, Pamela Bay, Katherine Boucher, Dylan Devino, Michael Eldredge, Samantha Frawley, Emma Hurley, Anne Kaufman, Danielle Semick, Mallory Sullivan, Sarah Zeger) and Columbia University undergraduate student, Ryan C. Noyes for assistance with milking and sample collection.

2.8 Abbreviations Used
BCFA- branched-chain fatty acids, C- Holstein-Jersey crossbreed, CLA- conjugated linoleic acids, DIM- days in milk, FA- fatty acid (s), FAME- fatty acid methyl ester (s), H- Holstein, J- Jersey, LA- linoleic acid, ALA- α-linolenic acid, MUFA- monounsaturated fatty acids, OA- oleic acid, OTU- operational taxonomic units, PA- palmitic acid, PUFA- polyunsaturated fatty acids, RA- rumenic acid, SA- stearic acid,
SFA- saturated fatty acids, TMR- total mixed ration, UFA- unsaturated fatty acids, VA- vaccenic acid

2.9 References


Table 2-1 Nutrient composition of the total mixed ration diet fed to Holstein, Jersey, and Holstein-Jersey crossbreed cows at 93, 183, and 273 DIM.

<table>
<thead>
<tr>
<th>Nutrient (% DM(^1)-basis)</th>
<th>Days in Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>93</td>
</tr>
<tr>
<td>%DM</td>
<td>40.7</td>
</tr>
<tr>
<td>CP(^2)</td>
<td>13.8</td>
</tr>
<tr>
<td>aNDFom(^3)</td>
<td>27.8</td>
</tr>
<tr>
<td>lignin</td>
<td>5.0</td>
</tr>
<tr>
<td>starch</td>
<td>22.4</td>
</tr>
<tr>
<td>sugar</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\(^1\) dry matter; DM; \(^2\) crude protein; CP; \(^3\) aNDFom-ash-corrected neutral detergent fiber; aNDFom.
Table 2-2 Fatty acid composition of the total mixed ration fed to Holstein, Jersey, and Holstein-Jersey crossbreed cows at 93, 183, and 273 days in milk.

<table>
<thead>
<tr>
<th>Fatty acid (% of total)</th>
<th>93</th>
<th>183</th>
<th>273</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.16</td>
<td>0.12</td>
<td>0.16</td>
</tr>
<tr>
<td>14:0</td>
<td>0.32</td>
<td>0.29</td>
<td>0.16</td>
</tr>
<tr>
<td>15:0</td>
<td>0.15</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td>16:0; PA</td>
<td>17.59</td>
<td>15.36</td>
<td>17.03</td>
</tr>
<tr>
<td>16:1 9c</td>
<td>0.46</td>
<td>0.42</td>
<td>0.41</td>
</tr>
<tr>
<td>17:0</td>
<td>0.23</td>
<td>0.16</td>
<td>0.21</td>
</tr>
<tr>
<td>18:0; SA</td>
<td>2.65</td>
<td>2.20</td>
<td>2.37</td>
</tr>
<tr>
<td>18:1 9t</td>
<td>0.06</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>18:1 9c; OA</td>
<td>19.52</td>
<td>22.87</td>
<td>19.88</td>
</tr>
<tr>
<td>18:1 11c; VA</td>
<td>2.54</td>
<td>3.40</td>
<td>2.68</td>
</tr>
<tr>
<td>18:2 9c,12c (n-6); LA</td>
<td>39.07</td>
<td>42.59</td>
<td>41.61</td>
</tr>
<tr>
<td>20:0</td>
<td>0.67</td>
<td>0.58</td>
<td>0.62</td>
</tr>
<tr>
<td>18:3 6c,9c,12c (n-6)</td>
<td>0.03</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>18:3 9c,12c,15c (n-3); ALA</td>
<td>14.40</td>
<td>10.03</td>
<td>11.56</td>
</tr>
<tr>
<td>20:1 8c (n-12)</td>
<td>0.08</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>21:0</td>
<td>0.05</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>20:2 11c,14c (n-6)</td>
<td>0.06</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>22:0</td>
<td>0.66</td>
<td>0.56</td>
<td>0.69</td>
</tr>
<tr>
<td>22:1 13c (n-9)</td>
<td>0.06</td>
<td>0.04</td>
<td>0.52</td>
</tr>
<tr>
<td>20:4 5c,8c,11c,14c (n-6)</td>
<td>0.03</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>23:0</td>
<td>0.20</td>
<td>0.17</td>
<td>0.22</td>
</tr>
<tr>
<td>24:0</td>
<td>0.86</td>
<td>0.74</td>
<td>0.88</td>
</tr>
<tr>
<td>24:1 15c</td>
<td>0.07</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>22:5 4c,7c,10c,13c,16c (n-6)</td>
<td>0.09</td>
<td>0.04</td>
<td>0.21</td>
</tr>
<tr>
<td>Σ SFA²</td>
<td>23.54</td>
<td>20.30</td>
<td>22.70</td>
</tr>
<tr>
<td>Σ MUFA³</td>
<td>22.79</td>
<td>26.93</td>
<td>23.73</td>
</tr>
<tr>
<td>Σ PUFA⁴</td>
<td>53.68</td>
<td>52.77</td>
<td>53.57</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>14.40</td>
<td>10.03</td>
<td>11.56</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>39.28</td>
<td>42.74</td>
<td>42.00</td>
</tr>
</tbody>
</table>

1 palmitic acid; PA, stearic acid; SA, oleic acid; OA, vaccenic acid; VA, linoleic acid; LA, α-linolenic acid; ALA, total saturated fatty acids; sum of SFA (12:0 through 24:0), 3 total monounsaturated fatty acids; sum of MUFA (16:1 through 24:1), 4 total polyunsaturated fatty acids; sum of PUFA (18:2 through 22:5)
Table 2-3 Protozoal fatty acid composition (% of total fatty acids) in primiparous Holstein (H), Jersey (J), and Holstein-Jersey crossbreeds (C) at 93, 183, 273 days in milk.

<table>
<thead>
<tr>
<th>Period</th>
<th>Breed</th>
<th>93 DIM</th>
<th>183 DIM</th>
<th>273 DIM</th>
<th>SEM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>J</td>
<td>C</td>
<td>H</td>
<td>J</td>
</tr>
<tr>
<td>12:0</td>
<td></td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>iso-14:0</td>
<td>0.11</td>
<td>0.32</td>
<td>0.32</td>
<td>0.24</td>
<td>0.24</td>
<td>0.20</td>
</tr>
<tr>
<td>14:0</td>
<td></td>
<td>0.71</td>
<td>0.72</td>
<td>0.60</td>
<td>0.62</td>
<td>0.60</td>
</tr>
<tr>
<td>iso-15:0</td>
<td>0.40</td>
<td>0.41</td>
<td>0.38</td>
<td>0.44</td>
<td>0.38</td>
<td>0.33</td>
</tr>
<tr>
<td>anteiso-15:0</td>
<td>1.07</td>
<td>0.88</td>
<td>0.98</td>
<td>1.00</td>
<td>0.91</td>
<td>0.87</td>
</tr>
<tr>
<td>15:0</td>
<td></td>
<td>0.99</td>
<td>1.21</td>
<td>1.01</td>
<td>1.33</td>
<td>1.01</td>
</tr>
<tr>
<td>iso-16:0</td>
<td>0.60</td>
<td>1.37</td>
<td>0.82</td>
<td>1.16</td>
<td>1.26</td>
<td>1.20</td>
</tr>
<tr>
<td>16:0</td>
<td></td>
<td>26.45</td>
<td>30.32</td>
<td>26.40</td>
<td>32.26</td>
<td>28.83</td>
</tr>
<tr>
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<td>0.26</td>
<td>0.20</td>
<td>0.32</td>
<td>0.21</td>
<td>0.23</td>
</tr>
<tr>
<td>anteiso-17:0</td>
<td>0.71</td>
<td>0.83</td>
<td>0.53</td>
<td>1.04</td>
<td>0.66</td>
<td>0.73</td>
</tr>
<tr>
<td>17:0</td>
<td></td>
<td>0.28</td>
<td>0.31</td>
<td>0.27</td>
<td>0.28</td>
<td>0.27</td>
</tr>
<tr>
<td>18:0</td>
<td></td>
<td>36.25</td>
<td>27.55</td>
<td>32.68</td>
<td>23.73</td>
<td>28.65</td>
</tr>
<tr>
<td>18:1 6-8t</td>
<td>0.28</td>
<td>0.25</td>
<td>0.27</td>
<td>0.18</td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td>18:1 9t</td>
<td></td>
<td>0.26</td>
<td>0.21</td>
<td>0.25</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>18:1 10t</td>
<td>0.55</td>
<td>0.36</td>
<td>0.48</td>
<td>0.17</td>
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<tr>
<td>18:1 11t</td>
<td>3.81</td>
<td>4.12</td>
<td>3.49</td>
<td>4.31</td>
<td>4.31</td>
<td>3.74</td>
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<tr>
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<td></td>
<td>9.77</td>
<td>11.12</td>
<td>9.93</td>
<td>12.47</td>
<td>12.80</td>
</tr>
<tr>
<td>18:1 6-8c/13:0</td>
<td>2.29</td>
<td>1.50</td>
<td>1.48</td>
<td>1.67</td>
<td>1.40</td>
<td>1.54</td>
</tr>
<tr>
<td>18:1 13c</td>
<td>1.23</td>
<td>0.94</td>
<td>1.14</td>
<td>0.67</td>
<td>0.91</td>
<td>1.10</td>
</tr>
<tr>
<td>18:2 9c/12c</td>
<td>5.34</td>
<td>6.03</td>
<td>5.56</td>
<td>8.79</td>
<td>7.67</td>
<td>7.77</td>
</tr>
<tr>
<td>18:3 9c/12c/15c</td>
<td>0.85</td>
<td>0.87</td>
<td>0.86</td>
<td>1.24</td>
<td>0.97</td>
<td>1.18</td>
</tr>
<tr>
<td>20:0</td>
<td></td>
<td>0.40</td>
<td>0.32</td>
<td>0.37</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>18:2 9c/11r</td>
<td>1.37</td>
<td>2.36</td>
<td>1.23</td>
<td>0.91</td>
<td>2.04</td>
<td>1.14</td>
</tr>
<tr>
<td>unknown DA</td>
<td>2.18</td>
<td>3.24</td>
<td>2.32</td>
<td>3.85</td>
<td>2.56</td>
<td>2.61</td>
</tr>
<tr>
<td>unknown FA</td>
<td>1.83</td>
<td>1.66</td>
<td>1.77</td>
<td>0.92</td>
<td>1.09</td>
<td>1.52</td>
</tr>
<tr>
<td>Total SFA</td>
<td>52.01</td>
<td>63.26</td>
<td>65.22</td>
<td>62.52</td>
<td>65.16</td>
<td>64.59</td>
</tr>
<tr>
<td>Total iso</td>
<td>1.59</td>
<td>2.07</td>
<td>1.08</td>
<td>1.94</td>
<td>1.86</td>
<td>1.78</td>
</tr>
<tr>
<td>Total anteiso</td>
<td>1.84</td>
<td>1.70</td>
<td>1.51</td>
<td>2.04</td>
<td>1.56</td>
<td>1.60</td>
</tr>
<tr>
<td>Total OCFA</td>
<td>4.98</td>
<td>6.08</td>
<td>7.79</td>
<td>5.75</td>
<td>4.99</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>Breed A</td>
<td>Breed B</td>
<td>Breed C</td>
<td>Breed D</td>
<td>Breed E</td>
<td>Breed F</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Total BCFA</td>
<td>3.23</td>
<td>4.10</td>
<td>5.91</td>
<td>4.22</td>
<td>3.78</td>
<td>4.70</td>
</tr>
<tr>
<td>Total 18:1 t</td>
<td>18.20</td>
<td>18.56</td>
<td>17.03</td>
<td>19.66</td>
<td>20.22</td>
<td>17.62</td>
</tr>
</tbody>
</table>

1dimethyl acetal; DA, fatty acids; FA, palmitic acid; PA, stearic acid; SA vaccenic acid; VA, oleic acid; OA, linoleic acid; LA, α-linolenic acid; ALA, conjugated linoleic acid; CLA, saturated fatty acid; SFA; odd-chain FA (OCFA), branched-chain FA (BCFA), 2breed; (B), days in milk; (DIM), and breed by days in milk interaction (B x DIM), Differences between breed, DIM and breed x DIM were declared significant at P<0.05.
Table 2-4 The shift in rumen protozoal fatty acid profiles in Holstein (H), Jersey (J), and Holstein-Jersey crossbreed (C) cows at 93,183, and 273 days in milk.

<table>
<thead>
<tr>
<th>Fatty acid (% of total)</th>
<th>Days in Milk</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>93</td>
<td>183</td>
<td>273</td>
<td></td>
</tr>
<tr>
<td>iso-15:0</td>
<td>0.40 b</td>
<td>0.38 b</td>
<td>0.50 a</td>
</tr>
<tr>
<td>15:0</td>
<td>1.07 b</td>
<td>1.08 b</td>
<td>1.27 a</td>
</tr>
<tr>
<td>iso-16:0</td>
<td>0.93 b</td>
<td>1.20 a</td>
<td>1.33 a</td>
</tr>
<tr>
<td>iso-17:0</td>
<td>0.24 b</td>
<td>0.25 b</td>
<td>0.34 a</td>
</tr>
<tr>
<td>anteiso-17:0</td>
<td>0.70 b</td>
<td>0.81 ab</td>
<td>0.97 a</td>
</tr>
<tr>
<td>17:0</td>
<td>0.29 b</td>
<td>0.29 b</td>
<td>0.35 a</td>
</tr>
<tr>
<td>18:1 6-8t</td>
<td>0.27 a</td>
<td>0.23 a</td>
<td>0.19 b</td>
</tr>
<tr>
<td>18:1 9c; OA</td>
<td>10.30 b</td>
<td>11.89 a</td>
<td>8.16 c</td>
</tr>
<tr>
<td>18:1 13c</td>
<td>1.11 a</td>
<td>0.99 b</td>
<td>0.83 b</td>
</tr>
<tr>
<td>18:2 9c,12c; LA</td>
<td>5.68 b</td>
<td>8.08 a</td>
<td>8.30 a</td>
</tr>
<tr>
<td>18:3 9c,12c,15c; ALA</td>
<td>0.86 c</td>
<td>1.13 b</td>
<td>1.72 a</td>
</tr>
<tr>
<td>18:2 9c,11t; RA</td>
<td>1.67 a</td>
<td>1.38 a</td>
<td>0.79 b</td>
</tr>
<tr>
<td>21:0</td>
<td>0.27 a</td>
<td>0.19 b</td>
<td>0.14 b</td>
</tr>
<tr>
<td>24:0</td>
<td>0.19 ab</td>
<td>0.16 b</td>
<td>0.21 a</td>
</tr>
<tr>
<td>26:0</td>
<td>0.07 b</td>
<td>0.06 b</td>
<td>0.19 a</td>
</tr>
<tr>
<td>Total aiso</td>
<td>1.69 b</td>
<td>1.73 b</td>
<td>2.11 a</td>
</tr>
<tr>
<td>Total SFA</td>
<td>65.15 a</td>
<td>62.22 b</td>
<td>65.28 a</td>
</tr>
<tr>
<td>Total 18:1 t</td>
<td>17.93 b</td>
<td>19.16 b</td>
<td>15.23 a</td>
</tr>
</tbody>
</table>

1 vaccenic acid; VA, oleic acid; OA, linoleic acid; LA, α-linolenic acid; ALA, rumenic acid; RA. Least squares means within a row without a common letter differ.
### Table 2-5 Percent abundance of rumen protozoal genera from lactating Holstein (H), Jersey (J), Holstein-Jersey crossbreed (C) dairy cows at 93, 183, and 273 days in milk.

<table>
<thead>
<tr>
<th>Time point</th>
<th></th>
<th>93 DIM</th>
<th>183 DIM</th>
<th>273 DIM</th>
<th>SE</th>
<th>( P ) Value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breed</td>
<td>H</td>
<td>J</td>
<td>C</td>
<td>H</td>
<td>J</td>
</tr>
<tr>
<td>Diploma</td>
<td>0.07</td>
<td>0.42</td>
<td>1.16</td>
<td>0.16</td>
<td>1.82</td>
<td>1.57</td>
</tr>
<tr>
<td>Diploplastron</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Entodinium</td>
<td>78.48</td>
<td>39.28</td>
<td>45.30</td>
<td>38.26</td>
<td>14.16</td>
<td>27.09</td>
</tr>
<tr>
<td>Epidinium</td>
<td>0.48</td>
<td>7.43</td>
<td>7.07</td>
<td>22.13</td>
<td>8.38</td>
<td>10.12</td>
</tr>
<tr>
<td>Eudiplodinium</td>
<td>1.54</td>
<td>5.80</td>
<td>15.83</td>
<td>9.01</td>
<td>15.34</td>
<td>21.62</td>
</tr>
<tr>
<td>Metadinium</td>
<td>1.24</td>
<td>15.92</td>
<td>1.00</td>
<td>0.28</td>
<td>8.66</td>
<td>0.36</td>
</tr>
<tr>
<td>Ophryoscolex</td>
<td>2.61</td>
<td>1.17</td>
<td>1.54</td>
<td>2.45</td>
<td>1.32</td>
<td>4.43</td>
</tr>
<tr>
<td>Ostracodinium</td>
<td>0.11</td>
<td>1.84</td>
<td>0.61</td>
<td>0.09</td>
<td>0.30</td>
<td>0.14</td>
</tr>
<tr>
<td>Polyplastron</td>
<td>2.75</td>
<td>0.92</td>
<td>1.22</td>
<td>0.42</td>
<td>0.76</td>
<td>0.62</td>
</tr>
<tr>
<td>Unclassified&lt;sup&gt;1&lt;/sup&gt;</td>
<td>12.83</td>
<td>25.44</td>
<td>23.64</td>
<td>24.43</td>
<td>46.50</td>
<td>31.51</td>
</tr>
<tr>
<td>Isotricha</td>
<td>0.53</td>
<td>1.41</td>
<td>1.67</td>
<td>1.85</td>
<td>2.57</td>
<td>2.12</td>
</tr>
<tr>
<td>Dasytricha</td>
<td>0.31</td>
<td>0.38</td>
<td>0.97</td>
<td>0.92</td>
<td>0.20</td>
<td>0.40</td>
</tr>
</tbody>
</table>

<sup>1</sup>Unclassified Ophryoscolecidae, <sup>2</sup>breed; (B), days in milk; (DIM), and breed by days in milk interaction (B x DIM), Differences between breed, DIM and breed x DIM were declared significant at \( P<0.05 \).
Table 2-6 Protozoal operational taxonomic unit (OTU)-based diversity measurements from lactating Holstein, Jersey, Holstein-Jersey crossbreed dairy cows at 93, 183, and 273 days in milk.

<table>
<thead>
<tr>
<th>Diversity Measure¹</th>
<th>Days in Milk</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>93</td>
<td>183</td>
<td>273</td>
</tr>
<tr>
<td>OTU</td>
<td>2.58 b</td>
<td>2.64 b</td>
<td>3.75 a</td>
</tr>
<tr>
<td>Good's coverage (%)</td>
<td>99.98</td>
<td>99.99</td>
<td>99.99</td>
</tr>
<tr>
<td>Shannon Diversity index</td>
<td>1.16x10⁻³ b</td>
<td>3.02x10⁻³ b</td>
<td>4.91x10⁻³ a</td>
</tr>
<tr>
<td>Inverse Simpson index</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Chao I Richness estimator</td>
<td>3.67 b</td>
<td>3.29 b</td>
<td>5.15 a</td>
</tr>
</tbody>
</table>

¹Least squares means within a row without a common letter differ.
Figure 2-1 Rumen protozoal genera in Holstein, Jersey, and Holstein-Jersey crossbreed dairy cows at 93, 183, and 273 days in milk.

Panel A shows the more abundant rumen protozoal genera, Panel B shows the less abundant rumen protozoal genera. Least-squares means without a common letter differ ($P<0.05$).
Figure 2-2 Pearson correlation heatmap used to compare the abundance of rumen protozoal genera to rumen protozoal fatty acids in Holstein, Jersey, Holstein-Jersey crossbreed dairy cows.

1saturated fatty acids; SFA, fatty acids; FA; monounsaturated fatty acids, MUFA
Chapter 3: Influence Of Periparturient And Postpartum Diets On Rumen Methanogen Communities In Three Breeds Of Primiparous Dairy Cows.

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3.1 Abstract

Background

Enteric methane from rumen methanogens is responsible for 25.9% of total methane emissions in the United States. Rumen methanogens also contribute to decreased animal feed efficiency. For methane mitigation strategies to be successful, it is important to establish which factors influence the rumen methanogen community and rumen volatile fatty acids (VFA). In the present study, we used next-generation sequencing to determine if dairy breed and/or days in milk (DIM) (high-fiber periparturient versus high-starch postpartum diets) affect the rumen environment and methanogen community of primiparous Holstein, Jersey, and Holstein-Jersey crossbreeds.

Results

When the 16S rRNA gene sequences were processed and assigned to operational taxonomic units (OTU), a core methanogen community was identified, consisting of *Methanobrevibacter (Mbr.) smithii, Mbr. thaueri, Mbr. ruminantium,* and *Mbr. millerae.* The 16S rRNA gene sequence reads clustered at 3 DIM, but not by breed. At 3 DIM, the mean % abundance of *Mbr. thaueri* was lower in Jerseys (26.9%) and higher in Holsteins (30.7%) and Holstein-Jersey crossbreeds (30.3%) (*P*<0.001). The molar concentrations of total VFA were higher at 3 DIM than at 93, 183, and 273 DIM, whereas the molar proportions of propionate were increased at 3 and 93 DIM, relative to 183 and 273 DIM. Rumen methanogen densities, distributions of the *Mbr.* species, and VFA molar proportions did not differ by breed.

Conclusions
The data from the present study suggest that a core methanogen community is present among dairy breeds, throughout a lactation. Furthermore, the methanogen communities were more influenced by DIM and the breed by DIM interactions than breed differences.

**Keywords:** archaea, diversity, Holstein, Holstein-Jersey, Jersey, 16S rRNA gene, mcrA, volatile fatty acids

### 3.2 Background

In the United States, enteric methane emissions from ruminants are the second largest anthropogenic source of methane, contributing to 25.9% of all methane emissions and global warming [1]. Methane production caused by rumen archaea (i.e., methanogens) leads to a 2-12% net loss of the dairy cow’s gross energy intake [2]. This loss contributes to a significant economic loss for farmers as it increases the quantity of feed needed to meet milk production demands.

The rumen is an anaerobic environment that houses a microbiome consisting of bacteria, protozoa, fungi, phages, and archaea. The bacteria, protozoa, and fungi (e.g. yeast) ferment feedstuff consumed by the host, and produce VFA. Acetate, butyrate, and propionate, the predominant VFA in the rumen, are the main energy sources for the host animal. Fermentation byproducts such as carbon dioxide, formate, hydrogen gas, methanol, and methylamines are used by methanogenic archaea for methane production. The majority of the methane is eructated and exhaled out by the ruminant into the environment. For methane mitigation strategies to be successful, it is important to
identify factors that may influence the rumen environment and thus, affect the methanogen density and diversity.

In dairy cattle rumen digesta samples, methanogens belonging to the genus \textit{Methanobrevibacter} (\textit{Mbr.}) are the most abundant species and primarily use hydrogen and carbon dioxide as substrates for methanogenesis \cite{3–5}. Methanogens from the genera \textit{Methanosphaera} (\textit{Msp.}) and \textit{Methanosarcina} use methanol and methylamines as substrates and are less abundant in the rumen \cite{4}. Most \textit{Mbr.} species in the rumen branch into two taxonomic clades, consisting of \textit{Mbr. smithii}, \textit{Mbr. gottschalkii}, \textit{Mbr. millerae}, and \textit{Mbr. thaueri} (i.e., \textit{smithii-gottschalkii-millerae-thaueri} (SGMT) clade) or \textit{Mbr. ruminantium} and \textit{Mbr. olleyae} (\textit{ruminantium-olleyae} (RO) clade) \cite{4}. Previous 16S rRNA gene sequence clone library data suggest that dairy breed influences the RO and SGMT clade distributions in the rumen \cite{4}.

Holstein and Jersey dairy cattle are the two most common dairy breeds used in the United States. Holstein cows are recognized for their high milk production, whereas Jersey cows are recognized for their increased fertility and higher milk components. Additionally, there is global interest in Holstein-Jersey crossbreeds to compensate for the decreased fertility in Holsteins and milk production in Jersey cows. It has been demonstrated that first generation Holstein-Jersey crosses have dry matter intakes, milk yields and solids in between those measured in Holstein and Jersey cows, respectively \cite{6}.

The transition period from a diet high in neutral detergent fiber (NDF) to a diet high in starch is a challenge for lactating dairy cattle. Prior to parturition, the NDF
content in the diet is elevated, while after parturition the energy content is increased with higher starch and fat levels. Kumar et al. [7] showed no difference in archaeal Shannon diversity or taxa when cows were transitioned from a high-fiber pre-partum diet to a low-fiber post-partum diet, however no studies described the rumen methanogen community across a lactation period. When quantifying VFA, another study observed that concentrations of total VFA, acetate, and propionate were decreased during the transition period in comparison to 100 days in milk (DIM) [8].

Previous research focused on rumen bacteria in pre- and post-partum dairy cattle, but rumen methanogens have not been identified or quantified under these conditions. Furthermore, the rumen methanogens of Holstein and Jersey dairy cattle with different parities and DIM were identified with limited data generated from pooled PCR samples using clone libraries. The present study focused on Holstein-Jersey crossbreeds, used next-generation sequencing (NGS), and animals of the same age, DIM, and parity. Given previous investigations into the rumen methanogen community in relation to breed and what is known about transitioning dairy cattle from one diet to another, we hypothesized that the rumen methanogen diversity and rumen VFA proportions in primiparous dairy cattle are affected by both breed and DIM, while methanogen densities do not vary. The objectives of the present study were to (1) measure the rumen VFA, (2) use NGS techniques to identify rumen methanogens, (3) distribute the archaeal 16S rRNA gene sequence reads into operational taxonomic units (OTU), (4) quantify the rumen methanogens, and (5) correlate VFA with specific rumen methanogen taxa from each breed during early (3 DIM), peak (93 DIM), mid- (183 DIM), and late-lactation (273 DIM).
3.3 Results

The 16S rRNA gene sequence data set is accessible through NCBI’s Sequence Read Archive, under the study accession number [SRP058775].

3.3.1 Rumen Volatile Fatty Acids

Breed and breed by DIM differences in total VFA concentrations or in individual VFA molar proportions were not observed. Total VFA concentrations were highest at 3 DIM ($P<0.01$). Propionate proportions were lowest at 273 DIM and highest at 3 and 93 DIM ($P<0.05$). Relative to 3 DIM, acetate proportions were higher at 183 and 273 DIM ($P<0.001$). Isobutyrate and lactate proportions were highest at 273 DIM ($P<0.01$). Isovalerate proportions did not differ by DIM (Table 3-1).

3.3.2 Rumen Methanogen Densities

The rumen methanogen densities (log10 copy number of methyl-coenzyme M reductase A (mcrA) gene/mL whole rumen digesta) were not different by breed ($P=0.93$) or DIM ($P=0.25$). The mean and standard error (SE) of densities by breed were 6.51 ± 0.04 (Holsteins), 6.53 ± 0.04 (Jerseys), and 6.52 ± 0.04 for (Holstein x Jersey crossbreeds), while the densities by DIM were 6.48 ± 0.05 (3 DIM), 6.49 ± 0.05 (93 DIM), 6.61 ± 0.05 (183 DIM), and 6.49 ± 0.05 (273 DIM), respectively. No differences by breed or DIM were observed.

3.3.3 Bioinformatics Analyses of the Rumen Methanogen Community

After 8,248,879 raw 16S rRNA gene sequence reads were quality checked, a total of 1,822,214 sequences from 87 whole rumen digesta samples had a Phred score of 25 or greater. The final data set contained 1,683,569 non-chimeric 16S rRNA gene sequence reads. 16S rRNA gene sequence read lengths (not including dashes) ranged
from 357-390 bp with a mean length of 358 bp. The mean and SE of sequence reads per individual by breed were: 19,039 ± 2,667 (Holsteins), 19,096 ± 2,744 (Jerseys), and 19,947 ± 3,034 (Holstein x Jersey crossbreeds). There were 298,689 total unique sequences (17.7% of total reads) with 67,072 chimeras removed. The numbers of unique sequence reads for each time point was: 67,111 (3 DIM), 87,455 (93 DIM), 77,477 (183 DIM), and 66,646 (273 DIM). Principle coordinate analysis (PCoA) did not demonstrate clustering of methanogen communities by breed. However, rumen methanogen communities clustered at 3 DIM, but not at other time points (Figure 3-1).

All sequence reads belonged to the phylum Euryarchaeota. The SGMT and RO clades did not differ by breed or DIM (Table 3-2). The majority of the total sequences reads were related to four methanogen species, *Mbr. smithii*, *Mbr. thaueri*, *Mbr. ruminantium*, and *Mbr. millerae* (Table 3-2). Because there was a significant interaction between breed and DIM for *Mbr. thaueri*, *Mbr. millerae*, *Methanosphaera*, and Methanoplasmatales, breed differences at specific DIM are presented. At 3 DIM, the mean % abundance of *Mbr. thaueri* was lower in Jerseys (26.9%) and higher in Holsteins (30.7%) and Holstein-Jersey crossbreeds (30.3%) (*P*<0.001). At 93 DIM, a lower abundance of the species *Mbr. thaueri* was observed in Holsteins (24.5%, *P*<0.05) and Holstein-Jersey crossbreeds (19.4%, *P*<0.01) when compared to Jerseys (35.0%). The species, *Mbr. ruminantium* (*P*<0.05) was higher at 93 DIM than at 273 DIM. *Msp.* The less abundant methanogen species (<5%) *Mbr. gottschalkii* and *Mbr. woesei* also varied by DIM, but not by breed. At 93 DIM (*P*<0.05) and 273 DIM (*P*<0.001), the abundance of *Mbr. gottschalkii* was higher than at 3 DIM. *Mbr. woesei* (*P*<0.001) were more abundant at 3 DIM, whereas the order Methanosarcinales was more abundant at 183 DIM.
Less than 1% of total methanogen sequences were very distantly related to the following methanogen genera: *Methanoculleus, Methanobrevibacter Methanoplanus, Methanospirillum*, and *Methanosarcina*.

### 3.3.4 OTU-Based Analyses

The 16S rRNA gene sequence reads clustered into 403 (3 DIM), 383 (93 DIM), 590 (183 DIM), and 547 OTUs (273 DIM). Dairy breed and breed by DIM did not affect rumen methanogen diversity measures (Table 3-3). Good’s coverage, Shannon diversity index, and Inverse Simpson index were affected by DIM. The Inverse Simpson indices were highest at 3 and 183 DIM (P<0.01), while Good’s coverage and the Shannon Diversity indices were highest at 3 DIM (P<0.05). The most and least OTUs shared between all animals were at 93 and 273 DIM, respectively (Table 3-4).

The top four OTUs shared by all breeds and at each stage of lactation were related to the species *Mbr. smithii, Mbr. thaueri, Mbr. ruminantium*, and *Mbr. millerae*. The least abundant OTUs were related to *Mbr. wolinni, Mbr. gottschalkii, Mbr. olleyae, Mbr. arboriphilus, Msp. stadtmanae*, unclassified *Methanosarcina*, Methanoplasmatales, *Methanoculleus*, and *Methanolobus*. The majority of the sequence reads (98.7 ± 0.1 %) clustered into OTU 1-4. At 3, 93, and 183 DIM, the mean abundance of OTU 1 was 30.8 ± 2.0%, 30.9 ± 2.8%, and 31.7 ±1.6%, respectively. At 273 DIM, OTU 2 was most abundant with 36.1 ± 2.3%. No breed effects were observed for OTUs 1-4 and the least abundant OTUs. However, the abundance of OTU 1 was lowered (P<0.05) in Holsteins (28.8 ± 2.0%) when compared to Holstein x Jersey crossbreeds (34.9 ±2.0%). DIM did not affect the distribution of OTU 3 or the least abundant OTUs. The abundance of OTU
2 increased at 273 DIM ($P < 0.05$), while the abundance of OTU 4 increased at 3 DIM ($P < 0.05$).

### 3.3.5 Relationship between Methanogen Taxa and VFA

Notably, the SGMT and RO clades were negatively correlated ($r = -0.98$, $P < 0.0001$) (Figure 3-2). A negative correlation between Mbr. smithii and Mbr. ruminantium was observed ($r = -0.66$, $P < 0.0001$). The abundance of the order Methanosarcinales was positively correlated with the order Methanoplasmatales ($r = 0.81$, $P < 0.0001$). Several weak correlations were observed between most methanogen taxa and VFA (Figure 3-2). The species, Msp. stadtmannae was negatively correlated to lactate ($r = -0.34$, $P < 0.01$) and positively correlated to propionate ($r = 0.33$, $P < 0.01$). Propionate was positively and negatively correlated to Mbr. ruminantium ($r = 0.22$, $P = 0.04$) and Mbr. thaueri ($r = -0.27$, $P = 0.04$), respectively. Several correlations were observed between individual VFA. Acetate was negatively correlated to propionate ($r = -0.84$, $P < 0.001$), butyrate ($r = -0.42$, $P < 0.001$), and valerate ($r = -0.54$, $P < 0.001$) and positively correlated to isobutyrate ($r = 0.48$, $P < 0.001$). Propionate was negatively correlated to isobutyrate ($r = -0.66$, $P < 0.001$) and lactate ($r = -0.39$, $P < 0.001$) and positively correlated to valerate ($r = 0.50$, $P < 0.001$).

### 3.4 Discussion

The present study is the first to investigate the rumen methanogen community across a lactation period in three dairy cattle breeds. The purpose of this experiment was to provide more knowledge about the rumen methanogen community and rumen parameters at 3, 93, 183, and 273 DIM in Holstein, Jersey, and Holstein-Jersey crossbreeds. This study identified the core methanogen community with NGS
technologies, quantified rumen VFA and methanogen densities, and correlated rumen methanogen species to one another and to VFA.

VFA are the main energy source provided to lactating dairy cattle and are the by-products of carbohydrate fermentation by rumen bacteria, protozoa, and fungi. Generally, propionate is a precursor to glucose and is increased when animals are provided a high-starch diet or provided the ionophore, monensin. Relative to 183 and 273 DIM, proportions of propionate were increased at 3 and 93 DIM, suggesting a greater demand for glucose by the cow during early lactation. Although the animals were provided 0.06% monensin pre-partum versus 0.02% post-partum, it is not possible to correlate the increase in propionate at 3 and 93 DIM with this additive. An effect from monensin would be more plausible at 3 DIM, when the cows were transitioning from a pre-partum to a post-partum diet, but this would not explain why propionate was also increased at 93 DIM.

Furthermore, the increase in total VFA concentrations observed at 3 DIM suggests an increase in carbohydrate fermentation at the start of lactation. Johnson et al. [9] stated that the fermentation of fiber is favored, providing insight into why VFA concentrations were elevated at 3 DIM versus 93, 183, and 273 DIM. In contrast, Danielsson et al. [10] found that total VFA concentrations did not vary in cannulated mid-lactation dairy cattle consuming 500:500 and 900:100 g/kg dry matter forage to concentration diets.

The present study is the first to compare the methanogen densities in three breeds of dairy cattle and by DIM. In agreement with our hypothesis, the methanogen densities did not vary by breed or DIM. Previously reported methanogen densities (i.e.,
log10 mcrA gene copies) from bulls on high-fiber (9.02) and starch diets (9.07) [11] were higher than what was observed in our study [11]. However, differences between the methanogen densities were not observed between the two diet groups [11]. In a study by Zhou et al. [12], the use of an exogenous fibrolytic feed enzyme additive did not affect the methanogen densities, yet, affected the methanogen community and methane production of lactating Holstein cows. Therefore, it appears that methanogen densities are not markedly affected by these specific diet alterations.

Previous work in dairy [4, 7, 10] and beef cattle [11, 13] also showed the genus *Mbr* to be the most predominant genus. As methanogens belonging to the genus *Mbr* use the rumen fermentation byproducts, such as hydrogen and carbon dioxide as substrates for methanogenesis, it is thought that the high levels of these byproducts in the rumen enable these methanogens to thrive over other species that rely on scarce substrates such as methylamines, methanol, or acetate [14, 15].

Although *Mbr* is the most abundant archaeal genus in ruminants, there are several species that are distributed into two different phylogenetic clades (i.e., SGMT and RO). In the present study, the SGMT clade was the most dominant branch by breed and DIM. Both *Mbr. smithii* and *Mbr. thaueri* made up the majority of the SGMT clade, while *Mbr. ruminantium* made up the majority of the RO clade. Previous research, using the same archaeal forward primer (Met86F), suggested a difference between SGMT-RO clade distributions between Holstein and Jersey cows [4]. However, the study revealed several limitations in the interpretation of the results. Animals were not blocked by parity, DIM, or age, the PCR products were pooled by breed and a clone library was constructed for each breed, and a limited number of clones were sequenced. It is
conceivable that these variables, but not primer bias, may have contributed to the observed breed differences. Another study showed a prevalence of the RO clade in both corn-fed Hereford crossbreed and potato-fed Hereford feedlot cattle in Canada [13]. Finally, the present study showed a strong negative correlation between the two clades suggesting that ruminants possess either a high abundance of SGMT or of RO and that dairy breed and DIM do not impact these proportions.

Because the four methanogen species *Mbr. smithii, Mbr. thaueri, Mbr. ruminantium,* and *Mbr. millerae* were identified in each breed and at each DIM time point investigated, our data showed the presence of a core methanogen community. Jeyanathan et al. [16] identified a common methanogen community between Holstein-Jersey crossbreeds, sheep, and red deer. Finding a core rumen methanogen community will enable further investigations into targeting specific species that are key contributors to methane production. Future work could isolate these species and determine which species produces the most methane.

Three out of the four methanogen species in the present study were previously identified in both Holstein and Jersey cows, while *Mbr. thaueri* was not. Recently, *Mbr. thaueri* was identified with the same primer pair used in the present study, at a high abundance in wild impalas from South Africa [17]. Omission of *Mbr. thaueri* from previous studies could be due to lack of sequencing depth, or diet of the animal. The higher abundance of *Mbr. thaueri* in Jersey cows at 93 DIM (i.e., peak lactation) may be a result of a higher dry matter intake (DMI), but future studies are needed to draw a clear link between DMI, milk yield, and the rumen methanogen species identified. It is conceivable that *Mbr. thaueri* and the other three methanogen species persisted because
the rumen environment and the substrates created by bacteria, protozoa, and fungi enabled these methanogens to thrive.

Throughout the lactation period and by breed, the methanol-utilizing genus, *Msp.* was identified in low abundances. Its mean % abundance was highest around peak lactation (93 DIM) and lowest at 3, 183, and 273 DIM. Similarly, Kumar *et al.* [7] compared the methanogen diversity between Holsteins at four weeks before calving and 1-5 days after calving and observed no differences in the genera *Mbr* or *Msp.* At 1-5 DIM, the methanol-utilizing genus *Msp* was more abundant (4.5%) in primiparous Holsteins than those from the present study (<1%). Like in the present study, animals were stomach tubed 2-3 hours post-feeding and received a diet before calving with the same NDF content (44%) [7]. *Msp* is typically more abundant in animals consuming feeds with elevated pectin levels [14]. Although not analyzed, it is possible that the diet in the present study had a lower quantity of pectin.

Although both OTU and 16S rRNA gene sequence classifications identified a core methanogen community, certain methanogen species were more abundant at different DIM time points. Relative to 93, 183, and 273 DIM the proportions of *Mbr. millerae* and *Mbr. woesei* were highest at 3 DIM. While previous research has not focused on rumen methanogen communities pre- and post-partum, one study suggested that the highly cellulolytic anaerobic fungi were more prevalent in pre-partum dairy cows, while rumen protozoa were less prevalent [18]. This suggests that as the dairy cows transition to a diet typically fed post-partum, there is a shift in the rumen microbiome and likely in the methanogen community.
The OTU coverage in the present study was almost 100%, indicating a sufficient sampling effort. OTU distribution did not vary by breed or DIM and the majority of the sequences were distributed into four main OTU. The methanogen diversity in the present study was not influenced by breed, but by DIM. King et al. [4] reported a higher Shannon diversity index and number of OTUs in lactating Holstein cows than in Jersey cows. However, the limited number of sequences from the cloned libraries, pooled samples by breed, and parity may have influenced the results. According to Kumar et al. [7], multiparous Holstein cows exhibit a higher Shannon diversity index than primiparous.

The Shannon diversity indices (i.e., species evenness and abundance) from the present study were highest at 3 DIM, while the 16S rRNA gene sequences reads clustered during this time as well. At 93, 183, and 273 DIM the sequence reads were mixed in one cluster. Previous research also demonstrated that the Shannon diversity indices of methanogens increased in bulls on a high-fiber diet (0.95) and decreased with a high-starch diet (0.79) [11]. These data suggested that a high-fiber diet leads to a more diverse methanogen community when compared to a high-starch diet. In another study, the Shannon diversity of Holsteins at 4 weeks before calving and 1-5 days after calving did not differ, but was most likely because there was not enough time between sampling [7]. Belanche et al. [19] suggested that the increased amount of cellulose and other heteropolysaccharides in a diet high in fiber leads to a more diverse microbial community. Therefore, a more diverse bacterial, fungal, or protozoal community may provide different substrates that enable the presence of a more diverse methanogen community.
3.5 Conclusions

The data presented here are the first to characterize the rumen methanogen communities in three dairy cattle breeds across a lactation period. NGS produced over 1 million sequence reads and demonstrated that diversity was different at 3 DIM. Notably, a core methanogen community persisted and consisted of four species, *Mbr. smithii*, *Mbr. thaueri*, *Mbr. ruminantium*, and *Mbr. millerae*. These methanogens may play a significant role in methanogenesis and in the utilization of substrates from bacterial, protozoal, and fungal fermentation. However future work is required to better delineate these relationships. The SGMT-RO clades did not vary by breed or DIM, instead, the SGMT clade was dominant in all three breeds. Although our results show that breed does not affect the rumen methanogen taxa *per se*, more studies are needed to clarify if this finding is consistent in other geographic locations and in dairy cattle consuming varying diets.

3.6 Methods

3.6.1 Animal Sampling

From May 2013 to May 2014, 22 primiparous lactating dairy cattle (7 Holstein (H), 8 Jersey (J), and 7 first generation Holstein x Jersey crossbreeds (X)) were co-housed at the University of Vermont (UVM) Paul Miller Research Complex in South Burlington, VT. At 3 DIM, one Jersey was excluded from all data analyses because of post-partum health concerns. The Institutional Animal Care and Use Committee at the UVM approved all animal sample collection methods under protocol # 13-031. All animals calved within a 2-month period. At 3, 93, 183, and 273 DIM, whole rumen digesta samples (50 mL) were collected 2-3h post-feeding at 0900 h via stomach
intubation from each animal. To collect rumen samples, a flexible milk hose (2.54 cm diameter) was passed through a speculum to the esophagus and to the rumen. The hose was marked at 200 cm to indicate the approximate location of the rumen. Once the tube contacted the fiber mat and rumen sounds were heard, a 600 cc livestock drench gun (Labelvage, France) collected the digesta. Whole digesta samples were immediately frozen at -20°C to minimize microbial activity.

3.6.2 Diet

Prior to calving, all animals consumed a pre-partum total mixed ration (TMR) diet. Within 24 hours post-partum, each cow was transitioned to a diet that was higher in starch and lower in NDF in comparison to the diet fed pre-partum (Table 3-5). Through out the study, a 70:30 forage to concentrate TMR was fed. Prior to calving the forages included corn silage (51.2%), haylage (8.3%), hay (13.4%), and concentrate (27.2%). The concentrate provided pre-partum contained: Amino Max (Afgritech, Watertown, NY; 18.8%) (a mixture of 2.5% dry matter (DM) lysine, 0.9% methionine, and 16.3% other essential amino acids derived from canola and soybean meals) soybean hulls (16.1%), PastureChlor® (West Central, Ralston, IA; 16.9%) (0.5% DM Ca, 6.1% Mg, and 10.6% Cl), canola meal (15.7%), soybean meal (12.5%), SoyChlor® (West Central, Ralston, IA; 12.5%) (high rumen bypass soybean meal with 4.5% DM Ca, 2.8% Mg, and 10.3% Cl), calcium carbonate (4.4%), magnesium sulfate (1.3%), trace vitamins and minerals (0.7%), magnesium oxide (0.5%), sodium chloride (0.5%), and Rumensin® (Elanco Animal Health, Greenfield, IN; 0.06%). Both PastureChlor and SoyChlor are cation/anion supplements typically added to a pre-freshening dairy cow diet and are provided to prevent hypocalcemia (milk fever). After the animals calved, the forage
consisted of corn silage (52.3%), haylage (15.9%), and concentrate (31.8%). The concentrate provided post-partum contained: corn grain (24.6%), citrus pulp (19.1%), Amino Max (16.4%), soybean meal (16.4%), canola meal (10.9%), Amino Enhancer (Poulin Grain Inc., Newport, VT; 5.5%) (blood and feather meal-derived amino acids with 8.0% DM lysine and 1.1% methionine), calcium carbonate (2.5%), sodium sesquinate (2.2%), sodium chloride (1.2%), magnesium oxide (0.7%), trace mineral premix and vitamins (0.43%), zinc methionine (0.05%), and Rumensin® (0.02%). The ionophore, monensin (e.g. Rumensin) was provided in both diets to simulate the diet of a typical lactating dairy cow. Hook et al.[20] demonstrated that monensin does not alter the rumen methanogen diversity or density in lactating dairy cows. TMR samples were collected weekly for three consecutive days and composited at the end of each week. Because cows calved within two months of one another, the mean and the standard error of the diet provided before calving are reported. Cumberland Valley Analytical Services (Hagerstown, MD) analyzed the feed samples and provided nutrient composition data.

### 3.6.3 Volatile Fatty Acid Analysis

The whole rumen digesta samples were spun in a Beckman J2-21 centrifuge at 10,000 x g for 20 min at 4°C and the resulting supernatant was filtered through filter paper (Whatman Inc. Clifton, NJ) to remove debris. Subsequently, the samples were diluted 1:1 with 0.06 M oxalic acid containing 50 µM trimethyl acetic acid (internal standard) and analyzed for VFA by gas-liquid chromatography (Varian 3800 GC, Walnut Creek, CA) coupled with a flame ionization detector and a customized packed column (2m x 2mm ID glass) with 4% carbowax and 80/120 Carbopac B-DA (Sulpeco, Bellefonte, PA) with nitrogen as carrier gas (15 mL/min flow rate). The other gases were
purified air at 300 mL/min and hydrogen makeup gas at 30 mL/min. The column was operated at 175°C; the total run time was 25 min. Both the injector and detector temperature were kept at 200°C. The injection volume was 1 µL. The identification of VFA was based on retention times using software Star Chromatography v5 (Varian) and quantified for their concentrations using respective VFA standards. Results are expressed in mM VFA.

3.6.4 Microbial DNA Extraction

Across 4 time points (3, 93, 187, and 273 DIM), 87 individual whole rumen digesta samples were collected. The previously frozen samples were thawed overnight at 4°C. Each sample was vortexed for 30s to homogenize the sample and break up the solid particles that settled to the bottom of the conical tube. The microbial DNA was extracted using the repeated-bead beating plus column (RBB+C) method [21] and followed previously described procedures [17].

3.6.5 Real-time PCR Amplification

The log_{10} of the copy number of the mcrA gene per mL of rumen digesta was determined by real-time PCR, while each sample was amplified in triplicate. Each real-time PCR included: 12.5 µL of SYBR® Green Mix, 6.5 µL of double distilled water, 2.5 µL of the methanogen-specific primer pair, mcrA-F and mcrA-R [22], and 1 µL of either diluted template DNA (10 ng/µL), positive (mixture of microbial DNA extract from King et al. [4]) or negative controls (double-distilled water). The mcrA gene was amplified in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) under previously published conditions [22]. The five mcrA gene standards were created by serial dilutions of 10 ng/µL of purified PCR product. The range of concentrations for the standard curve...
was from 0.001-10 ng/µL. An acceptable standard curve had an R² value greater or equal to 0.997. Using the BioRad CFX Manager (v.3.0) a model equation of the standard curve \(y = mx+b\) was used, where “x” was the log of the starting quantity, “b” the y-intercept of the quantification value (Cq), and “m” the slope of the line for log starting quantity versus Cq values. Individual densities were calculated using previously established methods [23].

3.6.6 PCR Amplification of the 16S rRNA gene

The archaeal-specific primer pair, Met86F [24] and Met471R [17] were used to amplify the V1-V3 hypervariable regions of the 16S rRNA gene via PCR, following previously published procedures [17]. Purified archaeal amplicons (25 µL) were sent to Molecular Research DNA Laboratories (Shallowater, TX) and sequenced with the Illumina MiSeq version 3 NGS platform.

3.6.7 Bioinformatics Workflow Used to Analyze MiSeq Sequences

The program MOTHUR, version 1.33.3, was used to perform bioinformatics analyses in-house [25]. Each time period was analyzed separately because of the lack of computing power and memory. Prior to using MOTHUR, a Perl script was used to trim the sequences to 350 bp at the reverse primer and each sequence was quality checked. All sequences with a Phred quality score of 25 or above were kept for further analyses. The command, trim.seqs removed barcodes and created a file that identified which sample belonged to which sequences.

To determine the number of unique sequences in the data set, the command unique.seqs was used. A Needleman-Wunsch pairwise alignment and an aligned reference file of known rumen methanogen 16S rRNA gene sequences were used with
the command align.seqs to align the unique sequences. Chimeric sequences were
identified with UChime [26] and removed with MOTHUR.

In order to detect any bacterial 16S rRNA gene sequences, the sequences were
classified with the 16S rRNA reference Ribosomal Database Project (RDP) files provided
by MOTHUR. These files contained known bacteria and methanogen sequences from
kingdom to genus taxonomic levels. The files were modified to contain species-level
names. Any bacteria sequences (<0.01%) were removed. The online RDP Classifier was
used at a 95% confidence threshold to further quality check our sequences. Sequences
with an unknown root were removed. Taxonomy and FASTA files containing 765 known
archaeal species names and 16S rRNA gene sequences were used to classify the sequence
reads into taxonomic species. The command cluster.split, was used with a 2% cutoff to
cluster the 16S rRNA gene sequences into OTUs. Once OTUs were formed, they were
classified with the command, classify.otu. The command summary.single calculated
OTU-based alpha diversities, Shannon Diversity Index, Chao I richness estimator,
Inverse Simpson index, and Good’s coverage. The subsample parameter in MOTHUR
was used to analyze the same number of sequences per individual sample. Shared OTUs
within and between breeds were counted with get.sharedseqs. To correlate OTUs (e.g.,
OTU 1 to OTU 2 at 3 DIM), the otu.association command used a Pearson correlation.
The command, merge.file combined the FASTA files of unique sequences. A subsample
of 100,000 sequences was taken and the distances between sequences with a 2% cutoff
were calculated with dist.seqs. Once a phylip distance file was created, the clearcut
command created a phylogenetic tree file. The output from clearcut was used with Fast
Unifrac to perform a principal coordinate analysis (PCoA) [27]. The PCoA determined if sequences from each sample cluster were based on breed or DIM.

3.6.8 Statistical Analyses
All data were analyzed with the repeated measures ANOVA model in SAS 9.4 (SAS Inst. Inc., Cary, NC) with PROC MIXED. The model included breed, DIM, and breed by DIM interactions as fixed effects and used the Kenward-Roger method to determine the degrees of freedom. A Pearson correlation, to determine the relationship between VFA and methanogen taxa, was performed with the PROC CORR. The online data visualization tool, Plotly, was used to generate a heatmap of the correlation values (r). Statistical significance was declared at $P<0.05$ and trends were declared at $0.05 \leq P \leq 0.10$.

3.7 Declarations

3.7.1 List of abbreviations
DIM-days in milk, DM-dry matter, DMI-dry matter intake, H-Holstein, J-Jersey, Mbr-Methanobrevibacter, mcrA-methyl coenzyme M reductase A, Msp-Methanosphaera, NDF-neutral detergent fiber, NGS-next-generation sequencing, OTU-operational taxonomic unit, PCoA-principal coordinate analysis, RBB+C-repeated-bead beating plus column, RDP-Ribosomal Database Project, RO-ruminantium-olleyae, SGMT-smithii-gottschalkii-millerae-thaueri, TMR-total mixed ration, UVM-University of Vermont, VFA-volatile fatty acids, X-Holstein x Jersey crossbreed

3.7.2 Ethics approval and consent to participate
The present study was performed in accordance with the Institutional Animal Care and Use Committee at the University of Vermont under protocol # 13-031.
3.7.3 Availability of supporting data
The data sets that support the results of the present study are included within the journal article.

3.7.4 Competing Interest
The authors declare that they have no competing interests.

3.7.5 Author’s contribution
LMC and MB collected rumen digesta samples. LMC performed all DNA extractions, PCR, bioinformatics, and statistical analyses. MB and LMC analyzed and performed statistical analyses on the VFA data. ADGW, JK, and LMC designed the study. LMC, ADGW, and JK prepared/wrote the manuscript. All authors read and approved the final version of the manuscript.

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3.8 References


Table 3-1 Rumen volatile fatty acids from lactating Holstein, Jersey, Holstein-Jersey crossbreed dairy cows at 3, 93, 183, and 273 days in milk

<table>
<thead>
<tr>
<th>VFA (% total)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Days in Milk</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>93</td>
<td>183</td>
<td>273</td>
<td>SE</td>
</tr>
<tr>
<td>Acetate</td>
<td>65.30 c</td>
<td>66.71 b</td>
<td>67.75 ab</td>
<td>68.53 a</td>
<td>0.51</td>
</tr>
<tr>
<td>Propionate</td>
<td>18.55 a</td>
<td>18.01 a</td>
<td>16.07 b</td>
<td>14.71 c</td>
<td>0.41</td>
</tr>
<tr>
<td>Butyrate</td>
<td>9.98 ab</td>
<td>9.52 b</td>
<td>10.22 a</td>
<td>10.01 ab</td>
<td>0.27</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>0.83 b</td>
<td>0.86 b</td>
<td>0.87 b</td>
<td>1.06 a</td>
<td>0.03</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.93 ab</td>
<td>0.99 a</td>
<td>1.01 a</td>
<td>0.82 b</td>
<td>0.04</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>0.84 a</td>
<td>0.72 a</td>
<td>0.70 a</td>
<td>0.76 a</td>
<td>0.05</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.44 b</td>
<td>3.29 b</td>
<td>3.38 b</td>
<td>4.12 a</td>
<td>0.15</td>
</tr>
<tr>
<td>A:P&lt;sup&gt;2&lt;/sup&gt; ratio</td>
<td>3.59 c</td>
<td>3.80 c</td>
<td>4.26 c</td>
<td>4.69 a</td>
<td>0.12</td>
</tr>
<tr>
<td>Total VFA&lt;sup&gt;3&lt;/sup&gt; (mM)</td>
<td>144.47 a</td>
<td>112.8 b</td>
<td>118.7 b</td>
<td>105.2 b</td>
<td>6.41</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means are based on Holstein (n=7), Jersey (n=8), and Holstein-Jersey crossbreeds (n=7). Means within a row without a common letter differ (P<0.05);<sup>2</sup> acetate:propionate; <sup>3</sup>volatile fatty acids
Table 3-2 Classification of rumen methanogen 16S rRNA sequence reads to taxa from lactating Holstein, Jersey, Holstein-Jersey crossbreed dairy cows at 3, 93, 183, and 273 days in milk

<table>
<thead>
<tr>
<th>DIM2 Breed</th>
<th>3</th>
<th>93</th>
<th>183</th>
<th>273</th>
<th>SE</th>
<th>B</th>
<th>DIM</th>
<th>B x DIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanobrevibacter</td>
<td>99.30</td>
<td>96.15</td>
<td>99.42</td>
<td>99.23</td>
<td>0.99</td>
<td>NS</td>
<td>†</td>
<td>NS</td>
</tr>
<tr>
<td>Mbr. woesel</td>
<td>2.12</td>
<td>2.47</td>
<td>1.58</td>
<td>1.06</td>
<td>0.22</td>
<td>NS</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td>Mbr. smithii</td>
<td>28.55</td>
<td>28.09</td>
<td>35.72</td>
<td>32.72</td>
<td>9.99</td>
<td>NS</td>
<td>†</td>
<td>NS</td>
</tr>
<tr>
<td>Mbr. gottschalkii</td>
<td>0.04</td>
<td>0.02</td>
<td>0.05</td>
<td>0.25</td>
<td>0.07</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>Mbr. millerae</td>
<td>10.99</td>
<td>8.39</td>
<td>8.0</td>
<td>7.45</td>
<td>5.02</td>
<td>NS</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Mbr. theaueri</td>
<td>30.70</td>
<td>26.70</td>
<td>30.32</td>
<td>28.03</td>
<td>3.51</td>
<td>NS</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>SGMT Clade</td>
<td>70.29</td>
<td>63.15</td>
<td>74.49</td>
<td>68.45</td>
<td>5.81</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mbr. ruminantium</td>
<td>26.18</td>
<td>29.82</td>
<td>22.88</td>
<td>27.43</td>
<td>5.11</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mbr. oleae</td>
<td>0.36</td>
<td>0.37</td>
<td>0.19</td>
<td>0.47</td>
<td>0.08</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>RO Clade</td>
<td>26.54</td>
<td>30.02</td>
<td>23.07</td>
<td>27.9</td>
<td>5.5</td>
<td>NS</td>
<td>†</td>
<td>NS</td>
</tr>
<tr>
<td>Methanosphaera</td>
<td>0.41</td>
<td>0.43</td>
<td>0.42</td>
<td>0.41</td>
<td>0.1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Methanoplasmta</td>
<td>0.15</td>
<td>0.2</td>
<td>0.07</td>
<td>0.2</td>
<td>0.1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Methanosarcinales</td>
<td>0.08</td>
<td>1.33</td>
<td>0.08</td>
<td>1.16</td>
<td>0.51</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1Probability of the effects due to breed (Holstein, Jersey, and Crossbreed) and their interaction and comparison to time period (3, 93, 183, and 273 DIM).
2Days in milk (DIM), H= Holstein (n=7), J= Jersey (n=8), X= Holstein-Jersey crossbreeds (n=7), Breed (B), 3Mbr= Methanobrevibacter

**P≤0.001; **P≤0.01; *P≤0.05; †0.05 ≤ P ≤ 0.10; no significance (NS) P ≥ 0.10.
Table 3-3: Operational taxonomic unit-based diversity measurements from lactating Holstein, Jersey, Holstein-Jersey crossbreed dairy cows at 3, 93, 183, and 273 days in milk

<table>
<thead>
<tr>
<th>DIM Breed</th>
<th>3</th>
<th>93</th>
<th>183</th>
<th>273</th>
<th>SE</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OTU</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>23.7</td>
<td>34.3</td>
<td>28.9</td>
<td>20.0</td>
<td>1.4</td>
<td>NS</td>
</tr>
<tr>
<td>J</td>
<td>37.7</td>
<td>33.5</td>
<td>22.2</td>
<td>24.4</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>X</td>
<td>18.5</td>
<td>29.7</td>
<td>31.1</td>
<td>23.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coverage (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>99.8</td>
<td>99.8</td>
<td>99.4</td>
<td>99.7</td>
<td>&lt;0.1</td>
<td>NS ***</td>
</tr>
<tr>
<td>J</td>
<td>99.7</td>
<td>99.8</td>
<td>99.6</td>
<td>99.5</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>X</td>
<td>99.9</td>
<td>99.8</td>
<td>99.4</td>
<td>99.6</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td><strong>Shannon Diversity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>1.5</td>
<td>1.4</td>
<td>1.5</td>
<td>1.5</td>
<td>&lt;0.1</td>
<td>NS *</td>
</tr>
<tr>
<td>J</td>
<td>1.5</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>X</td>
<td>1.4</td>
<td>1.5</td>
<td>1.4</td>
<td>1.4</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td><strong>Inverse Simpson</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>3.8</td>
<td>3.1</td>
<td>3.2</td>
<td>3.1</td>
<td>0.1</td>
<td>NS **</td>
</tr>
<tr>
<td>J</td>
<td>3.6</td>
<td>3.3</td>
<td>3.5</td>
<td>3.3</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>X</td>
<td>3.4</td>
<td>3.0</td>
<td>3.7</td>
<td>3.2</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td><strong>Chao I estimator</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>52.0</td>
<td>68.4</td>
<td>82.0</td>
<td>54.1</td>
<td>4.2</td>
<td>NS</td>
</tr>
<tr>
<td>J</td>
<td>94.7</td>
<td>63.4</td>
<td>47.0</td>
<td>52.2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>X</td>
<td>32.1</td>
<td>59.2</td>
<td>61.7</td>
<td>54.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Probability of the effects due to breed (Holstein, Jersey, and Crossbreed) and their interaction and comparison to time period (3, 93, 183, and 273 DIM).
2. H= Holstein (n=7); J= Jersey (n=8), X= Holstein-Jersey crossbreed (n=7), Breed (B), Days in milk (DIM), *** P<0.001; ** P<0.01; * P<0.05; no significance (NS) P ≥ 0.05.
Table 3-4 Number of shared operational taxonomic units among and between lactating Holstein, Jersey, Holstein-Jersey crossbreed dairy cows at 3, 93, 183, and 273 days in milk.

<table>
<thead>
<tr>
<th></th>
<th>3 DIM</th>
<th>93 DIM</th>
<th>183 DIM</th>
<th>273 DIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holstein</td>
<td>13</td>
<td>16</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Jersey</td>
<td>12</td>
<td>14</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Holstein x Jersey</td>
<td>11</td>
<td>13</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Between H-J</td>
<td>12</td>
<td>13</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Between H-X</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Between J-X</td>
<td>10</td>
<td>14</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>All</td>
<td>10</td>
<td>13</td>
<td>11</td>
<td>8</td>
</tr>
</tbody>
</table>

1Operational taxonomic unit; 2Days in milk; 3H= Holstein (n=7); J= Jersey (n=8), X= Holstein-Jersey crossbreed (n=7), Breed (B)
Table 3-5 Chemical composition of the diets (% DM-basis) provided pre- and postpartum

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Days in Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-fresh</td>
</tr>
<tr>
<td>DM(^1)%</td>
<td>36.8 ± 1.2</td>
</tr>
<tr>
<td>CP(^2)</td>
<td>14.1 ± 0.3</td>
</tr>
<tr>
<td>aNDFom(^3)</td>
<td>35.0 ± 2.1</td>
</tr>
<tr>
<td>Lignin</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Starch</td>
<td>13.9 ± 4.0</td>
</tr>
<tr>
<td>Sugar</td>
<td>3.1 ± 1.1</td>
</tr>
</tbody>
</table>

\(^1\)DM- dry matter; \(^2\)CP-crude protein; \(^3\)aNDFom-ash-corrected neutral detergent fiber
Figure 3-1 Principal coordinate analysis (PCoA) of rumen methanogen 16S rRNA sequences across a lactation.

Legend: The PCoA demonstrates the clustering of 16S rRNA sequences from Holstein (n=7), Jersey (n=8), and Holstein-Jersey crossbreed (n=7) cows at 3, 93, 183, and 273 days in milk (DIM). Red squares represent 3 DIM, the blue circles represent 93 DIM, mint green triangles represent 183 DIM, and light green triangles represent 273 DIM.
Figure 3-2 Pearson correlation heatmap comparing the abundance of rumen methanogen taxa to rumen VFA.

Legend: The heatmap depicts correlations made between rumen methanogen taxa and VFA from primiparous Holstein (n=7), Jersey (n=8), and Holstein-Jersey crossbreed (n=7). acetate to propionate (A:P), Methanobrevibacter (Mbr.), Methanosphaera (Msp.) smithii-gottschalkii-millerae-thaueri (SGMT), ruminantium-olleyae (RO), volatile fatty acids (VFA).
Chapter 4: Pasture Inclusion Of Spring Annual Forages Alters Relative Abundances Of Prevalent Rumen Methanogens From Lactating Dairy Cows.

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4.1 Abstract

In the Northeastern U.S., biomass of cool-season grasses is decreased in early spring. One potential solution is to provide annual forages (AF), such as small grains, in addition to cool-season grasses to lactating dairy cows. The objectives of this study were to: 1) determine if AF consumption would alter the rumen archaeal populations, densities, and diversities and 2) identify correlations between rumen archaeal, bacterial, and protozoal taxa in lactating dairy cows. A 21d experiment with eight cows consuming cool-season grasses (control, CON) and eight cows consuming cool-season grasses plus AF (AF group) was performed. During d 18-21, AF comprised 7.3% of the diet, reached maturity, and were lower in quality than the cool-season grasses. Rumen archaeal densities and diversity measures did not differ by group. Relative abundances of *Methanobrevibacter (Mbr) millerae* were greater in AF (11.2%) cows than CON (8.5%) cows, while abundances of *Mbr. ruminantium* were lower in AF (9.3%) than CON (13.9%) cows. More correlations were identified between archaea and bacteria of the phylum Firmicutes than between protozoa. *Mbr. ruminantium* was positively correlated to the bacterial family Ruminococcaceae. These results suggest that AF consumption changes the relative abundances of prevalent rumen archaea and that archaea may have specific and non-specific relationships with bacteria and protozoa, respectively.

4.2 Introduction

In the Northeastern US, early spring is a period of low pasture production. Providing adequate pasture is important to organic dairy farmers who are required to provide a 120d grazing season within a calendar year [1]. Of the total US organic dairies, 80% are located in the Northeast and Upper Midwest, illustrating the impact of the
Northeast region on the organic dairy industry [2]. Therefore, it is important to identify strategies to maintain milk production and feed efficiency during periods of low pasture production. One solution to overcome typical periods of decreased pasture mass and growth is to incorporate AF, such as spring available cereal forages or warm-season grasses into cool-season grass pasture.

The rumen of a dairy cow is characterized by the co-occurrence of all three domains of life: Archaea (e.g., methanogens), Bacteria and Eukarya (e.g., ciliate protozoa and fungi). Bacteria and Eukarya ferment feedstuff into volatile fatty acids (VFA) to meet the energy demands of lactation and generate hydrogen and carbon dioxide as major by-products. If hydrogen continues to increase in the rumen, it inhibits fermentation and metabolism. Rumen methanogens act as hydrogen sinks and maintain normal rumen function by utilizing by-products of microbial fermentation (e.g., H₂, CO₂, formate, and methanol) to produce methane gas, which is unusable to the animal. This co-occurrence enables rumen microbiota to form mutually beneficial relationships and a diverse rumen microbial ecosystem. Kittleman [3] suggested that there are highly-specific interactions between rumen methanogen and bacterial taxa, leading to microbial co-occurrence patterns.

In addition to their contributions to greenhouse gas emissions, enteric methane emissions contribute to a 2-12% decline in gross energy available for the cow [4], thus requiring farmers to provide additional feed to maintain milk production. It is important to recognize which factors may influence the rumen ecology, including the relative abundances of rumen methanogen species that may contribute to the development of methane mitigation strategies and improved feed efficiency. Several factors such as
breed, days in milk (DIM) [5], and age [6] alter the rumen methanogen community structures, but diet is the most influential determinant [7]. Previously, the consumption of a high-concentrate diet and ionophore supplementation [8,9], pre-and post-partum diets [5], and fiber-and starch-finishing diets [10] were shown to affect the rumen methanogen community structures in cattle. No studies, however, have demonstrated the effects of incorporating a mixture of AF on the rumen methanogen community structures and densities in lactating Jersey cows.

We hypothesized that: 1) the AF mixture of cereal forages contains a different nutrient composition than a traditional cool-season grass pasture, thereby altering substrate availability (e.g., H₂ and CO₂) and consequently the rumen methanogen community structure and 2) specific rumen methanogen species co-occur with rumen bacterial and protozoal taxa. The objectives of the study were to determine the effects of AF offered as a pasture mixture with traditional cool-season grasses and legumes on rumen methanogen densities, diversities, and co-occurrence with specific bacterial and protozoal taxa in lactating Jersey cows.

4.3 Methods and Materials

4.3.1 Study Design

The University of New Hampshire’s Institutional Animal Care and Use Committee (protocol #150302) approved all animal sample collection methods. From May-June 2015, sixteen lactating Jersey cows used in the study were co-housed at the University of New Hampshire Burley-Demeritt Organic Dairy Research Farm (Lee, NH) and divided into two treatment groups. Cows were matched by DIM, parity, and milk production. The control group (CON; n = 8) was provided a traditional cool-season grass
and legume pasture plus total mixed ration (TMR) and the second group (AF; n = 8) consumed a traditional cool-season grass and legume pasture with AF plus TMR. AF included barley (*Hardeum vulgare*), rye (*Secale cereale*), triticale (*x Triticosecale*), wheat (*Triticum spp.*), and hairy vetch (*Vicia villosa*). Before the start of the 21d experiment, CON cows averaged 83 ± 50 DIM, 3.1 ± 1.6 lactations, and produced 20 ± 4.5 kg milk/d, while AF cows averaged 86 ± 44 DIM, 2.9 ± 1.1 lactations, and produced 19 ± 2.7 kg milk/d.

**4.3.2 Diet**

Cows were offered a 60:40 forage:concentrate TMR after AM milking at 0800 h and pasture at 1600 h until the following AM milking at 0600 h. On an as-fed basis, TMR contained organic corn grain (55.0%), wheat middlings (18.3%), soybean meal (7.6%), cane molasses (3.4%), Redmond salt© (3.0%), canola meal (2.5%), ground barley (6.9%), calcium carbonate (1.8%), magnesium oxide (0.6%), Diamond V XP™ (*Saccharomyces cerevisiae*, 0.5%), Green Mountain Feeds Dairy Premix (0.1%), magnesium sulfate (0.1%), and sodium bicarbonate (0.1%), while forage included mixed grass-legume baleage. Each day, cows were rotated to their respective paddocks, which were next to the one previously grazed. The grass and legume pasture consisted of Kentucky bluegrass (*Poa pratensis*) orchardgrass (*Dactylis glomerata*), timothy grass (*Phleum pratense*), and white clover (*Trifolium repens*). AF pastures were 30% strip-tilled with a mixture of AF (barley, rye, triticale, wheat, and hairy vetch) and formulated to be 10% of the total DMI.

Pasture and TMR samples were collected for four consecutive days (d18-21) and composited into single pasture and one TMR samples for nutrient analyses. TMR and total pasture samples were analyzed by Dairy One (Ithaca, NY), while botanical
compositions (cool-season grasses and AF mixture) were analyzed by Cumberland Valley Analytical Services (Hagerstown, MD). Pre-grazing samples (n = 60) were collected from CON and AF pasture, thoroughly mixed, and divided into quarters. One quarter was used for dry mater (DM) determination; one quarter was used for botanical composition estimations and divided into mixed grasses, legumes, broadleaf weeds, and annual forage crops (AF treatment only) and the remaining two quarters were frozen at -20°C for subsequent analyses. Feedstuff samples were dried in a forced-air oven at 65°C for 48 h. TMR was provided to each cow via the Calan doors system (America Calan Inc., Northwood, NH), which allowed for individual DMI measurements.

The methods established by Bargo et al.[11] used in vitro DM digestibility to estimate individual pasture DMI. Briefly, cows consumed 1 kg of pelleted concentrate with chromium oxide (6.23 g/d) for 10 consecutive days. During the last week of the experimental period, individual fecal samples were collected quantitatively, twice daily immediately after AM and PM milkings for five consecutive days (d17-21). The DMI (kg/d) and nutrient components (% DM basis) were used to estimate the daily intakes of nutrients (e.g., crude protein, CP) from TMR and pasture.

4.3.3 Microbial DNA Analyses
Immediately after AM milking on d20 and d21, whole rumen digesta samples were collected via esophageal intubation and frozen at -80°C until further analysis. Purified microbial DNA was extracted from 250 µL of whole rumen digesta by the repeated-bead beating method [12]. The methanogen densities (log_{10} mcrA gene copies/mL of whole rumen digesta) were determined by previously described real-time PCR methods [5,13]. For taxonomic classification analyses, the methanogen primer pair
amplified the V1-V3 hypervariable regions of the 16S rRNA gene, while the protozoal-
specific primer pair 316F [18] and GIC758R [19] amplified the V3-V4 hypervariable
regions of the 18S rRNA gene on a BioRad C1000 Thermal Cycler (Hercules, CA) under
previously described conditions (methanogens and bacteria [15], protozoa [20]). Gel
extracted amplicons (4-6 total bands) were purified with the Qiaquick Gel Extraction Kit
(Qiagen, Venlo, Netherlands) and 5 µL of each purified amplicon was quality checked on
a 1% agarose gel. Once a clear bright band was observed amplicons (25 total µL, ≥ 15
ng/µL) were sent to Molecular Research Laboratories (Shallowater, TX) for paired-end
sequencing with the Illumina MiSeq (v.3) sequencer (Illumina Inc., San Diego, CA).

4.3.4 Bioinformatics Workflow

Raw archaeal and bacterial 16S, and protozoal 18S rRNA gene sequence reads
were analyzed in-house with the bioinformatics program, MOTHUR (v. 1.33) [21]. The
command trim.seqs quality checked the sequences (Q ≥ 25), trimmed the sequences to
350-500 bp, and removed sequences with greater than 8 homopolymers. All unique
sequences were aligned using the Needleman-Wunsch pairwise alignment algorithm with
a +1 match reward and penalties of -1 and -2 for mismatches and gaps, respectively. The
program UCHIME [22] detected potential chimeric sequences, which were removed from
the data sets. Taxonomic classification of sequences was determined with the
classify.seqs command using previously described reference taxonomy and FASTA files.
A subsample of 5,000 sequences per sample was taken before distance and operational
taxonomic unit (OTU)-based analyses. The command dist.seqs calculated pairwise
distances between aligned archaeal sequences. Archaeal 16S rRNA sequences were
clustered into OTU using the nearest-neighbor method with a 2% cutoff. OTU-based alpha diversity parameters Shannon, Inverse Simpson, and Good’s coverage were calculated with the command, summary.single. Distance matrices for Yue and Clayton [23] theta values were used to create the principal coordinate analysis (PCoA) of archaeal 16S rRNA gene sequences. RStudio (v. 3.2.1) was used to visualize the PCoA.

4.3.5 Statistical Analyses

One cow in the AF group was excluded from the microbial DNA analyses, as an adequate amount of rumen digesta was not obtained. A Welch’s unequal variance test was used to compare the means between CON and AF data. When variances were equal, a paired \( t \)-test, assuming equal variances, in JMP Pro 12 (SAS Institute Inc., Cary, NC) was used to test the experimental effect. All data are reported as least-squares means (LSM) ± the standard error mean (SE) with statistical significance declared at \( P < 0.05 \) and trends at \( 0.05 \leq P \leq 0.10 \). The CORR procedure in SAS (v. 9.4) used Pearson’s correlations to determine the relationships between archaeal taxa and other microbial taxa (protozoa and bacteria). The corrplot package in RStudio (v. 3.2.1) graphically displayed the correlation matrices. Linear regressions of relative abundances of specific microbial taxa were plotted in Prism6 (GraphPad Software Inc., La Jolla, CA).

4.4. Results

4.4.1 Diet Treatments

The nutrient composition (% DM) of TMR was 16.8% CP, 29.4% neutral detergent fiber (NDF), 21.9% acid detergent fiber (ADF), 3.1% lignin, and 26.8% starch. CON pasture included 16.0% CP, 53.3% NDF, 34.6% ADF, 3.8% lignin, and 0.8% starch, and AF pasture; 15.1% CP, 56.0% NDF, 32.1% ADF, 2.4% lignin, and 0.3% starch.
starch. Total DMI (TMR plus pasture) tended to be greater from CON (18.9 kg/d) than AF (18.1 kg/d, $P = 0.08$) cows (Table 4-1). Total estimated nutrient intakes of CP, ADF, lignin, and starch differed between CON and AF cows (Table 4-1).

The botanical composition of the CON pasture included on a DM-basis: 70% mixed grasses, 17% legumes, and 13% broadleaf weeds while that of the AF pasture included: 60% mixed grasses, 14% legumes, and 17% annual forage crops (13% small grains and 4% hairy vetch) and 9% broadleaf weeds. The nutrient content of the AF mixture included: 8.5% CP, 67.6% NDF, 5.7% lignin, and 2.6% starch, while that of the cool-season grasses contained 11.0% CP, 59.2% NDF, 4.8% lignin, and 3.2% starch.

**4.4.2 Rumen Archaeal Bioinformatic Analyses**

A total of 679,753 archaeal 16S rRNA gene sequence reads (350-450 bp) had a Phred score of 35 or greater. Ten percent of the total unique sequences were chimeric and removed from the data set. A total of 548,806 16S rRNA sequence reads (CON: 274,764 total sequences, AF: 274,042 total sequences) were classified to archaeal taxa. There was an average of 34,346 sequences from the CON and 39,149 sequences from the AF group. Quality-checked, non-chimeric sequence read lengths ranged between 348-398 bp with a mean length of 373 bp. All sequences had zero ambiguous bases and between 4-8 homopolymers.

Distances of 20,527 unique sequences were calculated at a 2% cutoff with the nearest-neighbor method and clustered into 272 OTU. On average, sequences from the CON group clustered into 23 OTU, while those from the AF group clustered into 24 OTU. Nineteen OTU were shared between the CON and AF groups, while 155 and 136 OTU were identified within the CON and AF groups, respectively. The Shannon
Diversity and Inverse Simpson indices did not differ between CON and AF cows (Table 4-2). The visualization of the PCoA demonstrated similarities between the majority of CON and AF sequences and dissimilarities between three individual samples and the data set (Figure 4-1).

4.4.3 Rumen Methanogen Diversity and Taxa
Rumen methanogen density (log_{10} methyl coenzyme A reductase (mcrA) gene copies/mL rumen digesta) did not differ between CON and AF cows (7.1 and 7.2, respectively) (Table 4-3). The majority of archaeal 16S rRNA gene sequences (96.3%) were classified to the genus *Methanobrevibacter* (*Mbr*). Less abundant archaeal genera (< 0.1% abundance) included those 16S rRNA gene sequences that were related to *Methanolobus, Methanoculleus, Methanosarcina, Methanospirillum, Picrophilus,* and *Thermoplasma*. Archaea belonging to the *Mbr. smithii-millerae-gottschalkii-thaueri* (SGMT) clade were more abundant in the AF (86.6%) than CON (81.7%, *P* = 0.02) cows, while archaea belonging to the *Mbr. ruminantium-olleyae* (RO) clade were less abundant in AF (9.6%) than CON cows (14.1%, *P* = 0.01). Archaea belonging to the species *Mbr. millerae* were more abundant in AF cows (11.2%) than CON cows (8.5%, *P* = 0.02), while those belonging to the species *Mbr. ruminantium* were less abundant in AF cows (9.3%) than CON cows (13.9%, *P* = 0.01). The SGMT:RO clade ratio was greater in AF (10.2) than CON (6.1) cows (*P* = 0.02, Table 4-3).

4.4.4 Co-occurrence of Rumen Archaeal and Microbial Populations
Amongst archaeal taxa, there were negative correlations between the occurrences of *Mbr. SGMT and RO clades (r = -0.98, *P* < 0.001) and *Mbr. ruminantium*
and *Mbr. thaueri* (*r* = -0.65, *P* < 0.01). The occurrences of the archaeal species *Mbr. smithii* and *Mbr. thaueri*, tended to be negatively correlated (*r* = -0.51, *P* = 0.05). The occurrence of the archaeal species *Mbr. millerae* was positively correlated with the protozoal genus *Epidinium* (*r* = 0.57, *P* = 0.02, Figure 4-2), while the occurrence of the archaeal species *Methanosphaera (Msp) stadmanae* was negatively correlated with the protozoal genus *Entodinium* (*r* = -0.63, *P* < 0.01, Figure 4-2).

Two correlations were observed between archaeal species and bacterial taxa of the phylum Bacteroidetes (Figure 4-3), while 18 correlations were observed between archaeal species and bacterial taxa of the phylum Firmicutes (Figure 4-4). The relative abundances of protozoal genera (Supplementary Table 4-4) and more abundant bacterial genera (*i.e.*, *Prevotella*) did not differ between CON and AF groups (Supplementary Table 4-5). The occurrence of the less abundant archaeal species *Mbr. woesei* was negatively correlated with the phylum Bacteroidetes (*r* = -0.54, *P* = 0.04, (Figure 4-3) and positively correlated with the phylum Firmicutes (*r* = 0.56, *P* = 0.03, (Figure 4-4)). The occurrence of the *Mbr. RO* clade was positively correlated to the occurrences of bacteria belonging to the families Ruminococcaceae (*r* = 0.54, *P* = 0.04) and unclassified Lachnospiraceae (*r* = 0.53, *P* = 0.04) and genera (> 1% relative abundances) *Oscillibacter* (*r* = 0.66, *P* < 0.01), and *Papillibacter* (*r* = 0.56, *P* = 0.03, (Figure 4-4)).

**4.5 Discussion**

**4.5.1 Diet**

As spring available AF were 17.0% of the total pasture DM, the proportion of broadleaf weeds in AF pasture was lower in comparison to CON pasture. The CON pasture biomass averaged 3,038 ± 303 and that of AF pasture was 4,052 ± 353 kg of.
DM/ha [24]. A greater biomass, however did not equate to a high-quality pasture. Because all cereal forages in the AF mixture reached maturity with seedhead emergence prior to the end of the 21d period, their nutrient contents were low quality (e.g., 8.5% CP and 68% NDF). The University of Vermont Extension Crops and Soil Program reported greater CP (15.4, 15.9, and 15.1% DM) and lower NDF (61.2, 60.9, and 60.1% DM) concentrations in barley, triticale, and wheat, respectively, harvested between boot and dough stages [25]. They were, however, grown as monocultures and not amongst other plant species. Several factors such as strip-tilling AF in already established legumes/mixed grasses pasture and weather (44 mm rain d15-d21, high: 24°C, low: 9°C, AccuWeather, Lee, NH) may have influenced the growth and maturity of the AF. Pitta et al. [26] demonstrated a high concentration of CP (21% DM) in vegetative stage wheat, which also caused mild cases of frothy bloat in steers. Therefore, future studies should focus on using high-quality cereal forages and increase their proportions on established pasture or feed them as monocultures earlier in the spring, while monitoring ruminants’ health.

4.5.2 Rumen Methanogen Taxa

In agreement with previous studies, the relative abundance of the Mbr. SGMT clade was greater than that of the RO clade, regardless of experimental group [5,27]. Although members of both phylogenetic clades utilize hydrogen and carbon dioxide as main substrates for methanogenesis, Mbr. ruminantium M1 lacks the isoenzyme, methyl coenzyme M reductase II (McrII), which is expressed at high ruminal concentrations of hydrogen [28]. Alternatively, Mbr. ruminantium contains the enzyme, methyl coenzyme M reductase I (McrI), which is expressed at low ruminal concentrations of hydrogen,
while other methanogen species, such as *Mbr. olleyae* strain DSM16632 and *Mbr. millerae* strain DSM16643 contain both McrI and McrII enzymes [29]. Once additional genome sequences of different *Mbr. ruminantium* and *Mbr. smithii* strains are completed, a better understanding about the greater prevalence of the SGMT clade in the rumen will be achieved.

Because both enzymes are present in the *Mbr. millerae* strain, this enables flexibility to grow in low and high hydrogen conditions, potentially contributing to its high abundance and the abundance of the *Mbr. SGMT* clade in the rumen. The greater and lower abundances of *Mbr. millerae* and *Mbr. ruminantium*, respectively in AF-fed and CON-fed cows suggest that the rumen of AF cows may have had greater concentrations of hydrogen. Further the *Mbr. SGMT:RO* ratio was greater in AF cows than in CON cows. This was likely influenced by plant maturity (i.e., cell wall carbohydrates) of the AF mixture and potential changes in redox potentials in the rumen between treatment groups. Friedman *et al.* [8] suggested that in addition to dietary components, such as fiber or starch, redox potentials are diet-derived factors that alter rumen microbial environments. This study also demonstrated that rumen methanogen species, including *Mbr. smithii* and *Mbr. ruminantium* have different numbers of anti-reactive oxygen species proteins, providing further evidence of species-specific growth requirements altered by different dietary regimes [8].

**4.5.3 Rumen Methanogen Diversity**

Good’s Coverage values indicated an adequate sampling effort from the subsample of 5,000 sequences per individual sample. The Shannon diversity indices from the CON (0.60) and AF-fed (0.80) cows were much lower than previously observed in
mid-lactation primiparous Jersey cows (1.40) [5] and multiparous Jersey cows (2.81) [32] fed TMR, and in steers offered either fiber or starch-based finishing diets (1.54, 1.58, respectively) [10]. Because the calculation of the Shannon diversity index in MOTHUR contains the number of observed OTU, the number of individual OTU, and the total number of individuals in the archaeal community, no change in the total number of OTU between CON and AF cows was expected [33]. Visualization of the theta Yu and Clayton distance matrices with the PCoA plot demonstrated that the ruminal 16S rRNA gene sequences from the CON and AF cows had similar genetic distances and did not cluster separately from each other. Three of the samples, however, clustered separately from the majority of the samples, which was influenced by inter-animal variations that included a greater number of individual OTU, Shannon diversity indices.

4.5.4 Co-occurrence of Rumen Archaea and Ciliate Protozoa
The association of rumen archaea with ciliate protozoa has been well-established [34–36]. Rumen archaea associate ecto- and endosymbiotically with rumen protozoa; using hydrogen generated by the protozoal hydrogenosomes for growth and methanogenesis [37]. Fluorescence in situ hybridization probing identified the archaeal species Mbr. smithii, Mbr. thaueri, and Msp. stadtmannae as major colonizers of Entodinium spp., regardless of forage type fed to dairy cows [36]. In the present study, it was not surprising that a negative correlation between the archaeal species Msp. stadtmannae and the protozoal genus Entodinium was observed as Msp. stadtmannae utilizes methanol, a by-product of pectin fermentation for methanogenesis and Entodinium spp. are amylolytic and produce hydrogen. A positive correlation between species belonging to Mbr. and the protozoal genus Entodinium was expected, but did not
occur. Henderson et al. [38] performed a global analysis of rumen microbiota from several ruminant species and proposed that the associations between archaea and protozoa were non-specific or occur rather at the strain-level. Therefore, further research should be conducted to determine if and what archaeal-protozoal associations are specific or non-specific.

4.5.5 Co-occurrence of Rumen Archaea and Bacteria

Rumen bacteria are the most diverse and abundant microbiota, which ferment feedstuffs into useable by-products for archaeal growth and methanogenesis. In the present study, only one correlation was observed between bacteria belonging to the phylum Bacteroidetes, while more correlations were identified between bacteria belonging to the phylum Firmicutes and archaeal species. Bacteria belonging to the phylum Bacteroidetes are associated with degradation of starch and sugars, while Firmicutes are associated with degradation of fiber [39,40]. However, Prevotella and Butyrivibrio spp., (Bacteroidetes) and Ruminococcus and Fibrobacter (Firmicutes) are capable of breaking down plant cell wall components [41] and producing hydrogen and carbon dioxide for methanogenesis.

Members of the RO clade and the archaeal species Mbr. ruminantium were positively associated with bacteria belonging to the families Ruminococcaceae and unclassified Lachnospiraceae, while members of the SGMT were negatively correlated. Kittelmann et al. [3] observed a positive correlation between Mbr. ruminantium and the bacterial family Fibrobacteraceae from three host ruminant species, including cattle. As Mbr. ruminantium M1 thrives in low hydrogen concentrations because it lacks the McrII isoenzyme [28], members of the families Ruminococcaceae, Lachnospiraceae, and Fibrobacteraceae could potentially produce lower concentrations of hydrogen than other
bacterial families. *Mbr. woesei* was positively correlated to the phylum Firmicutes, unclassified Lachnospiraceae and Ruminococcaceae bacterial families. An adhesion protein was identified in *Mbr. ruminantium* M1 that binds to hydrogen producing protozoa (*e.g.*, *Entodinium*) and the bacterial species *Butyrivibrio proteoclasticus* [42]. Further, a bacterial flagellum protein that caused an upregulation of genes involved in methanogenesis was also identified [43]. Future research should determine what other factors (*e.g.*, archaeal adhesions) contribute to the specific archaeal-bacterial associations.

**4.5.6 Conclusions**

In conclusion, the present study identified differences in the abundances of rumen archaeal species from lactating Jersey cows offered AF over a 21d period. The AF mixture matured quickly and was characterized by lower CP and higher NDF contents compared to the cool-season grasses. Notably, greater abundances of *Mbr. millerae* and lower abundances of *Mbr. ruminantium* in the AF than CON-fed cows may reflect the maturity of the AF mixture and differences in hydrogen concentrations and redox potentials in the rumen. Few correlations between archaeal and protozoal taxa indicated a non-specific relationship, whereas several correlations identified between archaeal and members of the bacterial phylum Firmicutes indicated a specific relationship. Future research is warranted to 1) characterize the rumen archaeal community structures in cows when AF are at different growth stages and 2) identify more adhesion and bacterial flagellar proteins to better understand microbe-to-microbe interactions, and 3) feed AF as a monoculture instead of a mixture.
4.6 Abbreviations

4.7 Competing Interest
The authors declare no competing interests in research.

4.8 Acknowledgements
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4.9 References


Z. Yu, M. Morrison, Improved extraction of PCR-quality community DNA from digesta and fecal samples., Biotechniques. 36 (2004) 808–12.


[38] D.W. Pitta, S. Kumar, B. Vecchiarelli, L.D. Baker, J.D. Ferguson, N. Thomsen, et al., Temporal dynamics in the ruminal microbiome of dairy cows during the


Table 4-1  Dry matter intakes of total nutrient components from total mixed ration plus pasture diets offered to lactating Jersey cows

<table>
<thead>
<tr>
<th>Component (kg/d)</th>
<th>CON</th>
<th>AF</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI</td>
<td>18.9</td>
<td>18.1</td>
<td>0.40</td>
<td>0.08</td>
</tr>
<tr>
<td>CP</td>
<td>3.18</td>
<td>2.91</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>NDF</td>
<td>7.69</td>
<td>7.31</td>
<td>0.21</td>
<td>ns</td>
</tr>
<tr>
<td>ADF</td>
<td>5.30</td>
<td>4.72</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>lignin</td>
<td>0.66</td>
<td>0.51</td>
<td>0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>starch</td>
<td>2.98</td>
<td>2.86</td>
<td>0.02</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

1 Cows fed a total mixed ration (TMR, 60:40 forage: concentrate), and mixed cool-season grasses and legumes (n = 8, CON). 2 Cows fed TMR and mixed cool-season grasses plus annual forage crops (n = 8, AF). 3 Significance declared at $P < 0.05$, non-significant (ns), 4 dry matter intake (DMI), 5 crude protein (CP), 6 neutral detergent fiber (NDF), 7 acid detergent fiber (ADF).
Table 4-2 Diversity and density of rumen archaeal 16S rRNA gene sequences from lactating Jersey cows offered legume/mixed grass or legume/mixed grass plus annual forage crop pastures

<table>
<thead>
<tr>
<th>Diversity Measures</th>
<th>CON(^a)</th>
<th>AF</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operational taxonomic units</td>
<td>23.25</td>
<td>24.29</td>
<td>3.55</td>
<td>ns</td>
</tr>
<tr>
<td>Good's Coverage (%)</td>
<td>99.59</td>
<td>99.60</td>
<td>0.00</td>
<td>ns</td>
</tr>
<tr>
<td>Shannon Diversity</td>
<td>0.60</td>
<td>0.80</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>Inverse Simpson</td>
<td>1.01</td>
<td>1.02</td>
<td>0.00</td>
<td>ns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Density</th>
<th>(\log_{10} \text{ mcrA gene copies/mL rumen digesta})</th>
<th>CON(^a)</th>
<th>AF</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.14</td>
<td>7.24</td>
<td>0.11</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Cows not offered annual forages (AF) (n = 8, CON), \(^b\) Cows offered AF (n = 7, AF), \(^c\) non-significant (ns), \(^d\) methyl coenzyme A reductase (mcrA)
Table 4-3 Taxonomic classification of rumen archaeal 16S rRNA gene sequences from lactating Jersey cows offered legume/mixed grass or legume/mixed grass plus annual forage crop pastures

<table>
<thead>
<tr>
<th>Taxon (relative % abundances)</th>
<th>CON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AF</th>
<th>SE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SGMT clade</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mbr. smithii</td>
<td>35.13</td>
<td>36.17</td>
<td>1.32</td>
<td>ns</td>
</tr>
<tr>
<td>Mbr. gottschalkii</td>
<td>0.04</td>
<td>0.07</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>Mbr. millerae</td>
<td>8.50</td>
<td>11.17</td>
<td>0.71</td>
<td>0.02</td>
</tr>
<tr>
<td>Mbr. thaueri</td>
<td>38.03</td>
<td>39.19</td>
<td>1.95</td>
<td>ns</td>
</tr>
<tr>
<td><strong>RO clade</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mbr. ruminantium</td>
<td>13.85</td>
<td>9.29</td>
<td>1.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Mbr. olleyae</td>
<td>0.29</td>
<td>0.26</td>
<td>0.05</td>
<td>ns</td>
</tr>
<tr>
<td>Mbr. woesei</td>
<td>2.57</td>
<td>2.16</td>
<td>0.15</td>
<td>0.07</td>
</tr>
<tr>
<td>Msp. stadtnanae</td>
<td>0.95</td>
<td>0.67</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Less Abundant Species</td>
<td>0.31</td>
<td>0.42</td>
<td>0.13</td>
<td>ns</td>
</tr>
<tr>
<td><strong>SGMT:RO clade</strong></td>
<td>6.06</td>
<td>10.24</td>
<td>1.14</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cows not offered annual forages (AF) (n = 8, CON), Cows offered AF (n = 7, AF), <sup>b</sup> Methanobrevibacter smithii-gottschalkii-milleriae-thaueri (SGMT), <sup>c</sup> Methanobrevibacter (Mbr), <sup>d</sup> ruminantium-olleyae (RO), <sup>e</sup> Methanosphaera (Msp). Significance declared at P < 0.05, non-significant (ns).
Supplementary Table 4-4 Relative abundances of rumen protozoal genera from lactating Jersey cows offered mixed legume/grass pasture and mixed legume/grass pasture plus annual forages

<table>
<thead>
<tr>
<th>Genus (relative % abundance)</th>
<th>CONa</th>
<th>AFb</th>
<th>SE</th>
<th>P-valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Diplodinium</em></td>
<td>6.64</td>
<td>6.30</td>
<td>0.88</td>
<td>ns</td>
</tr>
<tr>
<td><em>Diploplastron</em></td>
<td>3.60</td>
<td>2.42</td>
<td>0.57</td>
<td>ns</td>
</tr>
<tr>
<td><em>Entodinium</em></td>
<td>13.63</td>
<td>13.79</td>
<td>3.04</td>
<td>ns</td>
</tr>
<tr>
<td><em>Epidinium</em></td>
<td>1.61</td>
<td>0.96</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td><em>Eudiplodinium</em></td>
<td>43.04</td>
<td>49.34</td>
<td>6.43</td>
<td>ns</td>
</tr>
<tr>
<td><em>Metadinium</em></td>
<td>4.92</td>
<td>2.68</td>
<td>2.48</td>
<td>ns</td>
</tr>
<tr>
<td><em>Ostracodinium</em></td>
<td>24.48</td>
<td>22.71</td>
<td>5.34</td>
<td>ns</td>
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<tr>
<td><em>Polyplastron</em></td>
<td>1.42</td>
<td>1.20</td>
<td>0.25</td>
<td>ns</td>
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<tr>
<td><em>Dasytricha</em></td>
<td>0.30</td>
<td>0.26</td>
<td>0.07</td>
<td>ns</td>
</tr>
<tr>
<td><em>Isotricha</em></td>
<td>0.17</td>
<td>0.22</td>
<td>0.06</td>
<td>ns</td>
</tr>
</tbody>
</table>

a Cows not offered annual forages (AF) (n = 8, CON), b Cows offered AF (n = 7, AF), c Trends declared at 0.05 ≤ P ≤ 0.10, non-significant (ns).
Supplementary Table 4-5 Relative abundances of rumen protozoal genera from lactating Jersey cows offered mixed legume/grass pasture and mixed legume/grass pasture plus annual forages.

<table>
<thead>
<tr>
<th>Taxon (relative % abundance)</th>
<th>CON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SE</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes (p)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43.14</td>
<td>43.46</td>
<td>2.91</td>
<td>ns</td>
</tr>
<tr>
<td>Anaerophaga (g)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.83</td>
<td>0.91</td>
<td>0.09</td>
<td>ns</td>
</tr>
<tr>
<td>Barnesiella (g)</td>
<td>3.44</td>
<td>3.68</td>
<td>0.48</td>
<td>ns</td>
</tr>
<tr>
<td>Prevotella (g)</td>
<td>19.60</td>
<td>18.71</td>
<td>3.58</td>
<td>ns</td>
</tr>
<tr>
<td>Un-RF16 (f)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.98</td>
<td>2.31</td>
<td>1.49</td>
<td>ns</td>
</tr>
<tr>
<td>RC9 (g)</td>
<td>3.89</td>
<td>4.19</td>
<td>0.55</td>
<td>ns</td>
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<tr>
<td>Un-Bacteroidales (c)</td>
<td>2.55</td>
<td>2.79</td>
<td>0.34</td>
<td>ns</td>
</tr>
<tr>
<td>Un-Prevotellaceae (f)</td>
<td>2.45</td>
<td>2.57</td>
<td>0.19</td>
<td>ns</td>
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<tr>
<td>Xylanibacter (g)</td>
<td>5.82</td>
<td>5.82</td>
<td>0.40</td>
<td>ns</td>
</tr>
<tr>
<td>Firmicutes (p)</td>
<td>53.83</td>
<td>54.05</td>
<td>3.16</td>
<td>ns</td>
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<tr>
<td>Acetitomaculum (g)</td>
<td>2.16</td>
<td>2.39</td>
<td>0.36</td>
<td>ns</td>
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<tr>
<td>Butyrivibrio (g)</td>
<td>4.68</td>
<td>4.77</td>
<td>0.47</td>
<td>ns</td>
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<tr>
<td>Coprococcus (g)</td>
<td>1.41</td>
<td>1.25</td>
<td>0.11</td>
<td>ns</td>
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<tr>
<td>Hydrogenoanerobacterium (g)</td>
<td>3.94</td>
<td>3.81</td>
<td>0.30</td>
<td>ns</td>
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<tr>
<td>Incertae Sedis (g)</td>
<td>4.05</td>
<td>3.49</td>
<td>0.28</td>
<td>ns</td>
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<tr>
<td>Lachnospira (g)</td>
<td>2.73</td>
<td>2.17</td>
<td>0.19</td>
<td>0.07</td>
</tr>
<tr>
<td>Mogibacterium (g)</td>
<td>0.62</td>
<td>0.71</td>
<td>0.09</td>
<td>ns</td>
</tr>
<tr>
<td>Oscillibacter (g)</td>
<td>1.05</td>
<td>1.44</td>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>Papillibacter (g)</td>
<td>3.86</td>
<td>4.41</td>
<td>0.33</td>
<td>ns</td>
</tr>
<tr>
<td>Pseudobutyrivibrio (g)</td>
<td>1.13</td>
<td>0.81</td>
<td>0.21</td>
<td>ns</td>
</tr>
<tr>
<td>Robinsoniella (g)</td>
<td>4.27</td>
<td>4.10</td>
<td>0.16</td>
<td>ns</td>
</tr>
<tr>
<td>Ruminococcus (g)</td>
<td>0.83</td>
<td>0.70</td>
<td>0.13</td>
<td>ns</td>
</tr>
<tr>
<td>Syntrophococcus (g)</td>
<td>2.69</td>
<td>2.72</td>
<td>0.20</td>
<td>ns</td>
</tr>
<tr>
<td>Un-Clostridiales (c)</td>
<td>0.51</td>
<td>0.44</td>
<td>0.05</td>
<td>ns</td>
</tr>
<tr>
<td>Un-Lachnospiraceae (f)</td>
<td>7.81</td>
<td>8.98</td>
<td>0.86</td>
<td>ns</td>
</tr>
<tr>
<td>Un-Ruminococcaceae (f)</td>
<td>5.05</td>
<td>4.68</td>
<td>0.52</td>
<td>ns</td>
</tr>
<tr>
<td>Proteobacteria (p)</td>
<td>0.72</td>
<td>0.35</td>
<td>0.30</td>
<td>ns</td>
</tr>
<tr>
<td>Anaeroplasma (g)</td>
<td>1.18</td>
<td>0.86</td>
<td>0.26</td>
<td>ns</td>
</tr>
<tr>
<td>Total Uncultured Bacteria</td>
<td>0.35</td>
<td>0.39</td>
<td>0.04</td>
<td>ns</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cows not offered annual forages (AF) (n = 8, CON), <sup>b</sup>Cows offered AF (n = 7, AF), <sup>c</sup>Significance declared at P < 0.05, Trends declared at 0.05 ≤ P ≤ 0.10, non-significant (ns), <sup>d</sup>phylum (p), <sup>e</sup>genus (g), <sup>f</sup>family (f), unclassified (Un).
Figure 4-1 Principal coordinate analysis of rumen archaeal 16S rRNA gene sequences from lactating Jersey cows offered legume/mixed grass or legume/mixed grass plus annual forage crop pastures.

The principal coordinate analysis demonstrates the clustering of 16S rRNA gene sequences from CON (n = 8) and AF (n = 7) cows. Blue triangles represent the CON group and red circles represent the AF group.
Figure 4-2 Correlations between select rumen archaeal and protozoal taxa from lactating Jersey cows.

Pearson’s correlations between (a) the protozoal genus *Epidinium* and the archaeal species *Methanobrevibacter (Mbr) smithii* and (b) the protozoal genus *Entodinium* and the archaeal species *Methanosphaera (Msp) stadtmannae*. Significance declared at $P < 0.05$. 
Figure 4-3 Corrplot of Pearson’s correlations between prevalent rumen archaeal species and bacterial phyla Bacteroidetes from lactating Jersey cows

The corrplot depicts the correlations between prevalent archaea and genera belonging to the phylum Bacteroidetes. Significant correlations between archaeal and bacterial are highlighted in yellow. The legend values -1 to 1 are the Pearson’s correlation coefficients. *Methanobrevibacter smithii-gottschalkii-millerae-thaueri* (SGMT), *Methanobrevibacter* (*Mbr*), *ruminantium-olleyae* (RO), *Methanosphaera* (*Msp*), Unclassified (Un)
Figure 4-4 Corrplot of Pearson’s correlations between prevalent rumen archaeal species and bacterial phyla Firmicutes from lactating Jersey cows

The corrplot depicts the correlations between prevalent archaea and genera belonging to the phylum Firmicutes. Significant correlations between archaeal and bacterial taxa are surrounded by black squares. Significance declared at $P < 0.05$. The legend values -1 to 1 are the Pearson’s correlation coefficients. Methanobrevibacter smithii-gottschalkii-millerae-thaueri (SGMT), Methanobrevibacter (Mbr), ruminantium-olleyae (RO), Methanosphaera (Msp), Unclassified (Un)
Chapter 5: Spring Annual Forages Offered To Mid-Lactation Jersey Cows Altered Milk Fatty Acid Contents Without Changing Key Rumen Microbial Fatty Acids And Taxa

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5.1 Abstract

In the Northeastern U.S., early spring (i.e., March-April) is characterized by reduced pasture mass. This is especially important to organic dairy farmers who must provide 120d of grazing to their cows. Bioactive fatty acids (FA), such as odd and branched-chain (OBCFA) in milk are positively linked to human health. The purpose of this study was to determine if annual forage supplementation alters the rumen ecology and thereby enhances the content of bioactive FA in milk from lactating Jersey cows. Cows consumed either a control (CON; grass/legume mixture, n = 8), or cool-season grass pasture 30% strip-tilled with annual forages (AF; barley, rye, triticale, wheat, hairy vetch, n = 8) for a 21d period. Total mixed ration comprised 57% of the dry matter intake (DMI). The proportion of AF mixture was 17% of total pasture DM. Intakes of total n-3 FA were greater in AF- than CON-fed cows, while total n-6 FA and oleic acid intakes were greater in CON-fed cows. No differences were observed in ruminal VFA, protozoal cell FA or taxa, while few differences were observed in bacterial cell FA and taxa. Milk contents of iso-15:0 and 17:0 per serving of whole milk were greater, while total BCFA tended to be greater from CON than from AF-fed cows ($P = 0.09$). Milk content of oleic acid per serving was lower in AF than CON-fed cows, while contents of de novo FA (i.e., 10:0, 12:0, and 14:0) per serving of whole milk were greater in AF-fed cows. Our findings demonstrated the challenges of using AF as a dietary strategy to increase milk bioactive FA and instead, demonstrated that postruminal FA synthesis additionally influenced the milk FA contents.
5.2 Introduction

Milk contains over 400 different fatty acids (FA)\(^1\). Several of these FA derive from bacterial biohydrogenation (e.g., 18:1 \textit{trans} isomers) and microbial cells (e.g., 15:0, \textit{aiso}-15:0, \textit{iso}-15:0) and have demonstrated bioactive effects important for the maintenance of human health\(^2,3\). Branched-chain FA (BCFA), mainly derived from rumen microbiota, were found to be greater in serum of non-obese than obese women\(^2\), while odd-chain FA (OCFA) were greater in cognitively healthy individuals compared to those with Alzheimer’s disease\(^3\). Because cows’ milk is unique in providing rumen-derived bioactive FA, there is interest in altering their diet to maintain or even enhance the milk FA profile. It is, therefore, important to test whether or not alternative feeding strategies can alter the rumen microbial community structures and therefore, the contents of milk FA provided to the consumer.

Rumen microbiota enable dairy cattle to convert fiber and non-fiber carbohydrates to energy for milk production. The rumen is an anaerobic environment, consisting of archaea, bacteria, fungi, protozoa, and phages. Bacteria, fungi, and protozoa supply 70% of the cow’s energy by fermenting feedstuff into volatile fatty acids (VFA)\(^4\). Rumen bacteria are the most abundant rumen microorganisms. They biohydrogenate diet-derived polyunsaturated FA (PUFA), including linoleic acid (LA, 18:2 \textit{c}9,\textit{c}12) and \(\alpha\)-linolenic acids (ALA, 18:3 \textit{c}9,\textit{c}12,\textit{c}15) into biohydrogenation intermediates, conjugated linoleic acids (e.g., CLA, 18:2 \textit{c}9,\textit{t}11) and vaccenic acid (VA, 18:1 \textit{t}11) and the end-product, stearic acid (SA, 18:0). Protozoa are less numerous than bacteria (10\(^4\)-10\(^6\) cells/mL rumen fluid, 10\(^10\)-10\(^11\)/mL, respectively)\(^5\) and potentially contribute to half of
the rumen microbial biomass.\(^6\) Protozoa do not biohydrogenate PUFA,\(^7\) but unlike bacteria, they engulf chloroplasts rich in PUFA\(^8\). Protozoal cells contain a greater PUFA content than bacterial cells, while bacterial cells have greater contents of BCFA and SA\(^9\).

In the Northeastern U.S., early-spring is a period of decreased growth of cool-season grasses (e.g., orchard and timothy grasses). Organic dairy farmers who are required to provide a total of 120d of pasture to their cows\(^10\) are concerned about maintaining milk production and providing sufficient forages to meet energy demands of their cows. One potential strategy to overcome this issue is to supplement cool-season grasses with more nutrient-rich annual forages (AF), such as cereal grains and legumes (barley and hairy vetch, respectively). Previous studies showed that wheat pasture with high crude protein contents (CP, 21% DM) altered more abundant rumen bacterial taxa and that cereal grains, such as barley and wheat contained high levels of LA (> 40% of total FA)\(^{11,12}\).

We hypothesized that differences in nutrient contents (e.g., CP and FA) between AF and cool-season grasses would alter prevalent rumen microbial taxa and their cellular FA and thereby enhancing the content of bioactive FA in milk. The main objective of this study was to determine if cool-season grass pastures, 30% strip-tilled with a AF mixture, would alter rumen microbial taxa and cellular FA, thus altering the milk FA profile and content per serving of whole milk from lactating Jersey cows. Specific objectives were to: (1) quantify forage, rumen bacterial, and protozoal FA, (2) quantify relative abundances of rumen bacterial and protozoal taxa, and (3) identify relationships between rumen microbial taxa and their cellular FA.
5.3 Methods and Material

5.3.1 Experimental Design and Diet

The University of New Hampshire’s Institutional Animal Care and Use Committee approved all sample collection methods under protocol 150302. The study consisted of a covariate period (d-4 and -3) relative to a 21d experimental period, with 14d of diet adaptation and sample collection between d18-21. Sixteen lactating Jersey cows were co-housed at the University of New Hampshire Burley-Demeritt Organic Research Farm from early-May 2015 to early-June 2015 and were divided into two treatment groups after the covariate period. Cows in the control group (CON, n = 8) were matched by DIM (85 ± 46 days in milk, DIM), parity (3.0 ± 1.3), and milk yield (19.3 ± 3.7 kg/d) to cows in the alternative forage group (AF, n = 8).

The diet consisted of a total mixed ration (TMR) with a 60:40 forage to concentrate ratio and pasture. Concentrate in TMR consisted of: corn grain ground (55.0%), wheat middlings (18.3%), soybean meal (7.6%), cane molasses (3.4%), salt (3.0%), canola meal solvent (2.5%), ground barley (6.9%), calcium carbonate (1.8%), magnesium oxide (0.6%), Diamond V XP (0.5%), Green Mountain Feeds Nuplex (0.1%), magnesium sulfate (0.1%), and sodium bicarbonate (0.1%). Forage contained 100% mixed grass-legume baleage. Pasture intake was estimated by using chromium oxide as an external biomarker. Cows consumed 1 kg/d of pelleted concentrate with chromium oxide (6.23 g/d) for 10 consecutive days and individual fecal samples collected and pooled twice daily for five consecutive days (d17-21). Pasture DMI was estimated using previously established methods (13). After AM milking (0800 h), cows were fed TMR, while after PM milking (1800 h), cows grazed a new paddock until the next AM milking.
(0600 h). CON and AF paddocks were next to each other and blocked off from each other by electric fences. CON paddocks contained a mixture of the following cool season grasses; orchardgrass (*Dactylis glomerata*), timothy grass (*Phleum pratense*), and Kentucky bluegrass (*Poa pratensis*), as well as, legumes; alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*). During the covariate period, cows consumed the CON pasture. AF paddocks were 30% strip-tilled with AF and contained a mixture of wheat (*Triticum* spp.), rye (*Secale cereale*), barley (*Hordeum vulgare*), triticale (*x Triticosecale*), and hairy vetch (*Vicia villosa*). The remaining AF pasture comprised the same cool-season grasses and legumes mixture as the CON pasture.

Pasture (*i.e.*, pre-grazing) and TMR samples were collected for four consecutive days (d18-21), frozen at -20°C, and later composited. A total of 60 pre-grazing samples were collected from the CON and AF paddocks. After collection, pasture samples were mixed and divided into four equal portions. One portion was used to determine the nutrient composition, another portion was used to determine the botanical composition, and a third quarter was used to determine dry matter (DM). The remaining quarter was frozen at -20°C for subsequent analysis. CON pasture samples were divided into grasses, legumes, and broadleaf weeds. AF pasture samples were divided into grasses, legumes, broadleaf weeds, and AF. Dairy One and Cumberland Valley Analytical Services (Hagerstown, MD) performed nutrient composition analyses on the TMR and individual pasture components, respectively. Samples were dried at 65°C for 48 h in a forced-air oven to determine percent dry matter of each sample.

5.3.2 Milk Sample Collection
Cows were milked twice daily at 0600 h and 1630 h. Milk samples were collected for four consecutive milkings (d19-21), including the two AM milkings (d20-21) during which whole rumen digesta samples were collected. Milk samples (50 mL) for milk fat analyses were preserved in 2-bromo-2-nitropropan-1,3-diol, pooled by cow, and analyzed by the method described by Resende et al.\textsuperscript{(14)} Individual milk samples (50 mL) for FA analyses were collected and stored at -20°C with no preservative added. Prior to the lipid extraction, the samples were composited by individual cow and milk production and spun at 17,800 x g at 8°C for 30 min. The top layer (\textit{i.e.}, cream) was collected and frozen at -20°C. Milk lipid extraction and FA analyses using gas-liquid chromatography (GLC) were performed as described by Bainbridge et al.\textsuperscript{(15)}. Milk FA content on a triacylglycerol (TAG)-basis was assumed to be 93.3 FA/TAG and the milk fat content reported as g/kg of individual FA accounted for individual milk production and milk FA yield\textsuperscript{(16)}. Milk FA content (g/100g FA) did not take into account animal performance, but was also calculated (Supplementary Table 5-6). Milk FA content per serving of 3.25% whole milk was determined according to the calculations reported by Bainbridge \textit{et al.}\textsuperscript{(17)}:

\[
\text{Fat (g)/serving} = (3.25/100) \times 244 \text{g fat/serving}
\]

\[
\text{FA/serving (mg)} = 7.93 \text{g fat/serving} \times 0.933 \times 1000
\]

\[
\text{Individual FA/serving (mg)} = 7,398.69 \text{mg FA/serving} \times [\text{FA proportion (g/100g)/100}]
\]

5.3.3 Whole Rumen Digesta Collection

Whole rumen digesta (solid and fluid) samples were collected after AM milking between 0600-0800 h via esophageal intubation in the covariate period and d20 and d21 of the experimental period. In the covariate period, 10 cows instead of 16 were intubated in compliance with the initial IACUC protocol. From the total sample, an aliquot of 15
mL was saved for microbial DNA extractions, 50 mL for VFA analyses, and 420 mL for microbial fractionation. Saliva was poured off from each rumen sample and 1.5 mL of 1% methyl cellulose was added to the rumen digesta for microbial fractionation to detach the microbial cells from forage. Whole rumen digesta was strained through a layer of cheesecloth to collect fluid for VFA analyses, to which 50% H$_2$SO$_4$ (vol/vol) was added, and the samples were frozen at -80°C. The West Virginia University Rumen Fermentation Profiling Laboratory (Morgantown, WV) used GLC to quantify the VFA molar concentrations (Supplementary Table 5-7).

5.3.4 Rumen Microbial Diversity

The repeated bead-beating method plus column method established by Yu and Morrison$^{(18)}$ was used to extract microbial DNA from 250 µL of whole rumen digesta. PCR amplified the 16S and 18S rRNA genes of rumen bacteria and protozoa, respectively. The bacterial primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’)$^{(19)}$ and 519R (5’-GWATTACCG CGGCKGCTG-3’)$^{(20)}$ targeted the 16S V1-V3 hypervariable regions, while the protozoal primers P-SSU-316F (5’-GCTTTTCGWTGGTAGTATT-3’)$^{(21)}$ and GIC758R (5’- CAACTGTCTCTATKAAYCG-3’)$^{(22)}$ targeted the 18S V3-V4 hypervariable regions. For PCR of bacteria, DNA extract was diluted to 10 ng/µL. Each PCR consisted of 50 µL and included: 2 µL of DNA extract, 31.5 µL of double-distilled water (ddH$_2$O), 10 µL of HF buffer (New England BioLabs, Ipswich, MA), 1 µL of deoxynucleotide (dNTP), 2.5 µL 27F, 2.5 µL of 519R, and 0.5 µL of Phusion DNA polymerase (New England BioLabs). Five µL of DNA extract (undiluted) and 28.5 µL of ddH$_2$O were used to amplify protozoa.
Bacterial amplicons were generated in a GeneAmp PCR Systems 9700 thermal cycler (Life Technologies, Carlsbad, CA) under the following conditions: hot start (98 °C, 4 min), 35 cycles of denaturation (98 °C, 10 s), annealing (56 °C, 30 s), extension (72 °C, 30 s). Protozoal amplicons were generated with a Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: a hot start (94 °C, 240 s), 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 60 s). The last cycle in both protocols included an extension at 72 °C for 6 min. DNA (2 µL) from bacterial or protozoal pellets were positive controls and ddH2O (2 µL) was the negative control. The Qiagen QIAquick Gel Extraction Kit (Hilden, Germany) extracted DNA amplicons from agarose gel bands and purified amplicons to concentrations between 15-20 ng/µL. Paired-end sequencing with the Illumina MiSeq (v.3) sequencing platform was performed at Molecular Research Laboratories (Shallowater, TX).

5.3.5 Bioinformatic Analyses Of Microbial Sequences Reads
Bioinformatic analyses were performed with the open-software program MOTHUR v. 1.33(23). All 16S and 18S rRNA gene sequence reads were analyzed using a previously described workflow(24). Trim.seqs removed the barcoded primers from the DNA sequences and set a minimum average quality score of 25 or greater. Bacterial and protozoal sequences were aligned using the Needleman-Wunsch pairwise alignment method with reference alignment files of known protozoal 18S rRNA gene sequence reads derived from Ishaq and Wright(22). In-house Perl Scripts previously described by Bainbridge et al.(25) aligned the conserved regions of 500 bacterial 16S rRNA gene sequence reads, which were used as a reference alignment file. Alignments were manually checked for any misaligned sequences. Unique sequences were checked for
chimeras with UChime\textsuperscript{(26)}. Previously established methods\textsuperscript{(25,27)} were used for taxonomic and diversity analyses of 5,000 (bacteria) and 10,000 (protozoa) sequences per sample.

5.3.6 Bacterial and protozoal cell FA analyses
The differential centrifugation procedure described by Or-Rashid \textit{et al.}\textsuperscript{(9)} and modified by Cersosimo \textit{et al.}\textsuperscript{(24)} were used to obtain rumen bacterial and protozoal fractions from individual cows. The cleaning step was repeated four times with the protozoal pellet. Live protozoa were viewed under a microscope to observe possible forage contamination (< 5%). Individual bacterial and protozoal pellets were stored at -80°C to stop all microbial activity and freeze-dried (Labconco, Kansas City, MO) for 48 h before lipids were extracted. Methods described by Cersosimo \textit{et al.}\textsuperscript{(24)} were used to extract lipids from bacterial and protozoal pellets, produce FA methyl esters (FAME) by transesterification\textsuperscript{(28)} and perform GLC analyses. Microbial cell FA compositions were determined by methods described by Bainbridge \textit{et al.}\textsuperscript{(15)}. Briefly, the oven temperature program included an initial temperature of 45°C held for 4 min, then programmed to increase by 13°C/min to 167°C with a 40 min hold, and programmed to increase by 4°C/min to 218°C with a 23 min hold at the end. Microbial dimethyl acetals were identified via GC-MS as described by Cersosimo \textit{et al.}\textsuperscript{(24)}.

5.3.7 Statistical Analyses
The MIXED procedure in SAS (v. 9.4) used the completely randomized block design to test the effect of treatment between CON and AF cows at the end of the 21d experimental period. The model included data from the covariate period. The CORR procedure in SAS determined the relationship between microbial taxa and cellular FA by calculating Pearson’s correlation coefficients (r), while the Corrplot package in R Studio
was used to visualize the correlations. All data are reported as the least-squares means ±
the standard error mean with trends defined at 0.05 ≤ P ≤ 0.10 and statistical significance
declared at P < 0.05.

5.4 Results

5.4.1 Dietary Nutrients And Fatty Acid Contents
Total DMI tended to be lower in AF (18.1 kg/d) than CON-fed cows (18.9 kg/d,
P = 0.08, Table 5-1). On a DM-basis, the AF mixture was 17% of total pasture DM. The
n-6/n-3 ratios of CON and AF pastures, and the AF mixture were 1.2, 0.5, and 2.7,
respectively (Table 5-2). Intakes of CP, neutral detergent fiber (NDF), and starch from
did not differ between treatment groups. Estimated pasture intakes of lignin and starch
from pasture by AF cows were 200 g/d and 60 g/d, respectively and 320 g/d and 20 g/d,
respectively, by CON-fed cows (P < 0.001). Total FA intake and intakes of OA and LA
were greater from CON than AF cows, while intakes of ALA were greater from AF than
CON cows (P < 0.001, Table 5-3).

5.4.2 Rumen Microbial Fatty Acid Compositions And Taxa
Stearic acid (SA, 18:0) and palmitic acid (PA, 16:0) were the most abundant FA
identified in bacterial and protozoa cells, while VA and oleic acid (OA, 18:1 c9) were the
most abundant UFA identified (Table 5-3). Total bacterial cell MUFA contents tended to
be lower in AF-fed (12.99 g/100g FA) than in CON-fed cows (14.43 g/100g FA, P =
0.06, Table 5-3). Bacterial 16:1 c11, 18:1 c12, and 18:1 c15 contents were 0.45 g/100g
FA, 0.68 g/100 g FA, and 0.75 g/100 g FA, respectively from CON-fed cows and 0.37
g/100 g FA, 0.55 g/100 g FA, and 0.70 g/100 g FA, respectively from AF-fed cows (P <
0.05).
Raw bacterial 16S and protozoal 18S rRNA gene sequence reads are accessible under the accession number SRP081268, in the Sequence Read Archive of the National Center for Biotechnology Information. The phyla Bacteroidetes and Firmicutes were the most abundant, while Proteobacteria was the third most abundant phylum, representing less than 1% of bacterial sequences. Relative abundances of bacterial phyla Firmicutes (CON: 53.8%, AF: 54.1%) and Bacteroidetes (CON: 43.1%, AF: 43.5%) did not differ between treatment groups. Prevalent bacterial classes included Bacteroidia (42.1%) and Clostridia (51.9%), Sphingobacter (1.0%), and Mollicutes (1.5%). Rumen bacteria of the orders Bacteroidales (42.1%) and Clostridiales (51.9%) were prevalent amongst all samples, however, did not differ between groups. Rumen bacteria of the families Prevotellaceae (27.5%), Lachnospiraceae (31.3%), and Ruminococcaceae (17.6%) were the most prevalent. The genus Prevotella was the most abundant, but relative abundance did not differ between CON (19.6%) and AF-fed cows (18.7%). The bacterial genus Oscillibacter was greater in cows fed AF (1.4%) than in CON cows (1.1%, \( P = 0.02 \)). Relative abundance of the genus Lachnospira tended to be lower in cows fed AF (2.2%) than in CON cows (2.7%, \( P = 0.07 \), Figure 5-1).

The majority of 18S rRNA gene sequence reads belonged to the order Entodiniomorphida (99.0%) and family Ophyroscoleidae, whereas the remaining belonged to the order Vestibuliferida (1.0%) and family Isotrichidae. No differences in protozoal cell FA or taxa were observed between CON and AF groups (Table 5-4). The genus Ostracodinium was the most prevalent, but the relative abundances did not differ between CON (24.5%) and AF-fed cows (22.7%). Relative abundances of the genus
Epidinium tended to be lower in cows fed AF (1.0%) than in cows fed CON (1.6%, \( P = 0.09 \), Figure 5-1A).

### 5.4.3 Milk Fatty Acid Contents

No differences were observed in the total milk de novo (<18 carbons), mixed (PA and 16:1 c9), or preformed (≥18 carbons) FA contents (g/kg milk) between CON and AF-fed cows (Table 5-5). Milk contents of ALA, LA, total BCFA, OCFA, and SFA did not differ between CON and AF groups (Table 5-5).

Contents of iso-15:0 and 17:0 per serving of whole milk were greater from CON-fed (19 mg and 44 mg, respectively) than from AF-fed cows (18 mg and 42 mg, respectively) (Table 5-5). Total n-3 FA content per serving tended to be greater in CON-fed (52 mg/serving) cows versus AF-fed cows (48 mg/serving) (\( P = 0.09 \)), while the total n-6 FA content per serving did not differ between the groups. In comparison to milk from AF-fed cows, there was a trend for a greater content of ALA per serving milk from CON cows (42 mg/serving vs. 39 mg/serving, \( P = 0.07 \)). Content of OA per serving was greater in CON cows (1197 mg/serving) than in AF cows (1111 mg/serving, \( P = 0.02 \)). Total MUFA content per serving was greater in CON (1651 mg/serving) cows versus AF cows (1548 mg/serving, \( P = 0.02 \)). Content of total SFA per serving was greater in AF (5432 mg/serving) than in CON cows (5329 mg/serving, \( P = 0.04 \)).

### 5.4.4 Correlations Between Microbial Taxa and FA

The bacterial genus Prevotella positively correlated with bacterial iso-15:0 (\( r = 0.53, P = 0.04 \)), and anteiso-15:0 (\( r = 0.81, P < 0.001 \)), and 15:0 (\( r = 0.67, P < 0.001 \)). Butyrivibrio tended correlated with 15:0 (\( r = -0.48, P = 0.07 \)) and correlated with anteiso:15:0 (\( r = -0.56, P = 0.03 \)). Lachnospira was negatively correlated with bacterial
iso-15:0 \( (r = -0.55, P = 0.03) \) and anteiso-15:0 \( (r = 0.60, P = 0.01) \), and 15:0 \( (r = -0.66, P < 0.01) \). Xylanibacter was positively correlated to bacterial LA \( (r = 0.60, P = 0.02) \) and ALA \( (r = 0.52, P = 0.048) \). The protozoal genus Entodinium tended to correlate with protozoal aiso-17:0 \( (r = 0.37, P = 0.08) \). Epidinium was negatively correlated with protozoal 15:0 \( (r = -0.45, P = 0.04) \) and Metadinium with iso-14:0 \( (r = -0.45, P = 0.04) \).

### 5.5 Discussion

In the present study, we investigated the influence of AF consumption on dietary, ruminal, and milk FA profiles by using GLC. Next-generation sequencing techniques identified bacterial and protozoal taxa and correlations demonstrated potential relationships of individual taxa to individual FA.

Firmicutes was the most abundant bacterial phylum identified in both treatment groups. Similarly, Holstein heifers consuming orchardgrass\(^{(29)}\) and dairy cows consuming cool-season grasses\(^{(30)}\) had greater abundances of Firmicutes than Bacteroidetes. In contrast, cows that consumed 100% TMR diets had greater abundances of Bacteroidetes than Firmicutes\(^{(25,31,32)}\). The seven most abundant bacterial taxa *Butyrivibrio, Prevotella, Ruminococcus*, Unclassified Ruminococcaceae, Unclassified Bacteroidales, Unclassified Clostridiales, and Unclassified Lachnospiraceae were previously considered to be part of a core rumen bacterial microbiome, yet were only 67% of the total bacterial sequences in the data set of 742 rumen fluid samples\(^{(33)}\). These seven taxa accounted for 43% of total bacteria \( (19\% \text{ Prevotella}) \) from Jersey cows in the present study and 78% of total bacteria \( (56\% \text{ Prevotella}) \) in Jersey cows consuming a 100% TMR diet at 183 DIM\(^{(25)}\) when the same primer pair and sequencing platform were used. Although the genus *Prevotella* is the most prevalent rumen bacterial genus regardless of diet, it is more abundant in
ruminants consuming diets with concentrate\(^{(33)}\). Previously, wheat at the vegetative stage contained greater CP contents (21% DM), which likely altered the rumen environment and caused frothy bloat\(^{(11)}\). Consumption of 100% wheat pasture by steers lead to a greater Bacteroidetes:Firmicutes ratio and lower acetate:propionate ratio\(^{(11)}\). Cereal forages and mixed grass/legume pastures in the present study reached maturity within the 21d experiment, and as expected with mature pasture, had lower CP contents (8.5% DM cereal forages, 16.0% CON, and 15.1% AF). Pasture quality, in addition to a short dietary adaption period, and lower inclusion rates of AF in comparison to previous studies, are potential sources that may have led to the few changes observed in the rumen bacterial communities between groups.

Because key bacterial phyla and genera did not differ between treatment groups, the suggestion by Vlaeminck et al.\(^{(34)}\) that differences in the BCFA and OCFA profiles exiting the rumen reflect the rumen bacterial population is plausible. *Prevotella* is the most abundant bacterial genus found in the rumen bacterial population and was previously shown to contain the greatest amount of *anteiso*-15:0 (36.7 g/100g FA) in comparison to *Butyrvibrio fibrosolvens* (16.2 g/100g FA), *Ruminococcus albus* (9.4 g/100g FA), and *Fibrobacter succinogens* (7.7 g/100g FA)\(^{(28,34)}\). Cellulolytic bacteria (*e.g.*, *Fibrobacter succinogens*) were suggested to contain greater amounts of *iso*-FA than *anteiso*-FA, while the opposite was shown for amylolytic bacteria (*e.g.*, *Prevotella*). In the present study, *Prevotella* positively correlated with bacteria *iso*-15:0 \((r = 0.53, P = 0.04)\) and *anteiso*-15:0 \((r = 0.81, P < 0.001)\). Despite the greater estimated intake of starch from the CON group, relative abundances of *Prevotella* and ruminal propionate did not differ between treatment groups. The amount of milk *iso*-15:0 per serving of
CON-fed cow’s milk was greater than the amount from AF-fed cows. Though
*Lachnospira*, a sugar and pectin fermenter, was more abundant in CON-fed than AF-fed
cows, it is unlikely that this genus caused the increase in milk *iso*-15:0. This genus was
less abundant relative to *Prevotella*, negatively correlated with bacterial *iso*-15:0, and
previously *Lachnospira multiparus* contained low contents of *iso*-15:0 (1.1 g/100g FA) in
comparison to *Ruminococcus flavefaciens* (35.7 g/100g FA)\(^{28,34}\). Instead, postruminal
modifications\(^{35}\), although not measured by this study were more likely the cause and
should be accounted for in future studies.

Few studies have identified the rumen protozoal community structures using
next-generation sequencing (NGS) techniques\(^{24,36–38}\). Kittelmann *et al.*\(^{36}\) and Tapio *et
al.*\(^{38}\) used 454-pyrosequencing and Illumina MiSeq platforms, respectively, and
reported that 18S rRNA gene sequences belonging to the amylolytic genus *Entodonium*
were the most prevalent protozoa identified. Cersosimo *et al.*\(^{24}\) used Illumina MiSeq to
characterize the rumen protozoal community in different dairy breeds and across a
lactation and found protozoa belonging to the genus *Entodonium* to have the highest
relative abundance, however this genus was more abundant in Holstein (45.2%) than
Jersey cows (23.3%) across a lactation. Jersey cows from the present study had lower
relative abundances of *Entodonium* (13.7%). Protozoa of the genus *Entodonium* engulf
large particulate matter containing starch granules\(^{6,39}\), but the greater estimated intake
of starch from CON-fed cows did not alter relative abundances of *Entodonium*.

Protozoal cells also contain OBCFA, research, however, has focused on their
UFA contents and their potential to increase flows of ALA and CLA to the duodenum
and mammary gland. *Epidinium* spp. contain chloroplasts in their cytoplasms, while
spinach chloroplasts from the more abundant species *Entodinium caudatum* were stored in the food vacuole and undergo phago-lysosomal fusion and digestion\(^{(40)}\). It has been suggested that cytoplasmic chloroplasts might be protected from digestive enzymes in protozoa\(^{(8)}\). *Epidinium* spp. and protozoal contents of ALA were greater in steers consuming fresh grass than hay\(^{(8)}\). Huws *et al.* noted that some ingested chloroplasts might not be available to rumen protozoa\(^{(41)}\). Although the total intake of ALA and total n-3 FA in AF-fed cows was estimated to be greater than CON-fed cows, it is possible that 1) the total n-3 FA content in the CON pasture was more bioavailable to the protozoa (*i.e.*, *Epidinium*) despite no treatment differences in protozoal cell ALA, 2) ALA content in the cereal grains was not accessible to the protozoa, or 3) the actual intake of ALA and total n-3 was greater from CON-fed cows as a result of individual selectiveness of various forage species on pasture. While milk ALA content per serving of milk tended to be greater in CON-fed than AF-fed cows, we suggest that this would not be enough to elicit a biological effect on human health. Cows from the present study provided greater contents of LA (141 mg and 103 mg, respectively) and ALA (50 mg and 36 mg, respectively) per serving of milk than from cows previously housed at the same farm under the same management practices\(^{(14)}\). However, the later group consumed 100% TMR, not 57%\(^{(14)}\).

OA is the most abundant UFA found in milk and has been linked to the prevention of cardiovascular diseases, partially through the mitigation of oxidative stress on cardiomyocytes\(^{(42)}\). Although contents of OA and total MUFA in milk were not different between treatment groups *per se*, their contents per serving of milk were greater from CON-fed than AF-fed cows. The estimated intakes of OA and SA from CON
pasture were greater than AF pasture, while the average OA content per serving of whole milk (1154 mg) was lower than the average OA per serving of U.S. retail whole milk (1745 mg). This is likely a result of lower amount of OA in pasture versus in TMR and breed differences between Holsteins and Jerseys\(^{17}\). Dietary OA is extensively biohydrogenated in the rumen to SA, however with incomplete biohydrogenation OA and 18:1 \textit{trans} isomers can exit the rumen\(^{43}\). As indicated by Loften \textit{et al.}\(^{44}\), both \textit{de novo} FA synthesis of 16:0 and desaturation of SA to OA in the mammary gland, make it difficult to quantify the amount of dietary FA transferred to milk. Milk OA is mainly derived by \(\Delta^9\) desaturase activity on SA in the mammary gland and enhances milk fluidity\(^{44}\). Enjalbert \textit{et al.}\(^{45}\) observed that the desaturation of SA to OA in the mammary gland of cows was 52% and desaturation also occurs in the duodenal mucosa. Though it is inconclusive if diet or postruminal synthesis caused greater contents of OA per serving CON milk than AF milk, OA is a bioactive FA linked to the maintenance of human health and potentially of interest to the consumer.

When describing milk saturated FA (SFA) it is important to identify the individual milk SFA instead of focusing on the total milk SFA contents. AF-fed cows had greater contents of medium-chain SFA, 8:0-14:0, per serving of whole milk than CON-fed, which is attributed to an increase in \textit{de novo} FA synthesis in the mammary gland. When cows consumed ryegrass/white clover pasture and grain supplementation (5 kg/d of a 75% barley, 25% wheat pellet), greater milk 10:0, 12:0, and 14:0 contents were observed than without supplementation\(^{46}\). Bacterial 17:0 tended to be greater in CON than AF-fed cows, while milk 17:0 per serving was greater in CON-fed cows. Chain-elongation of 15:0 in the mammary gland, however, potentially contributed to the latter
finding. Average contents of 15:0 (68 mg) and 17:0 (42 mg) per serving of whole milk from the present study were lower in comparison to mid-lactation Jersey cows consuming a 100% TMR diet (95 mg and 50 mg, respectively)(17), but similar to U.S. retail milk (66 mg and 39 mg, respectively)(47). The OCFA, 15:0 and 17:0, play key roles in increasing cell membrane fluidity and prostaglandin formation(48), and may prevent the risk of developing coronary heart disease(49) and multiple sclerosis(50). In addition, diet rich in lauric acid (12:0) has been shown to enhanced the serum lipid profile in healthy men and women(51).

In conclusion, consumption of AF had little influence on the rumen microbiota and their cellular FA compositions, however de novo FA, however, were greater in AF-fed cows than CON-fed cows. The AF diet yielded larger contents and estimated intakes of total n-3, yet, this did not translate into increased contents of total n-3 FA in protozoal cells or milk. The bioavailability of n-3 FA in the chloroplasts to the protozoa and the rapid biohydrogenation of n-3 FA by rumen bacteria contributed to this finding. Notably, content of OA was greater in the CON diet and milk per serving. Both dietary OA and desaturation of SA to OA in the mammary gland likely contributed to the milk content. Future studies are warranted to examine an increase of AF inclusion (e.g., 25% or 50% of diet) in the diet, measure duodenal flow of FA and postruminal FA synthesis, to potentially establish a positive effect on animal performance or rumen microbiota, while maintaining rumen health.

5.6 Acknowledgements
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5.7 Funding Sources
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5.8 Conflict of Interest
None.

5.9 Authorship
LC, RT, and KJ collected on-farm data. LC performed PCR, lipid extractions and analyses, bioinformatics, and wrote the manuscript. AB, LC, and JK contributed to the conception and design of the study. JK, AB, SG contributed to data interpretation and revised content of the article.

5.10 References


Table 5-1 Estimated dry matter and nutrient intakes of total mixed ration plus mixed grasses/legume pasture and total mixed ration plus mixed grass/legume pasture and annual forages offered to lactating Jersey cows

<table>
<thead>
<tr>
<th>Component (kg/d)</th>
<th>CON(^a)</th>
<th>AF(^b)</th>
<th>SE</th>
<th>P-value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMR</td>
<td>10.9</td>
<td>10.7</td>
<td>0.09</td>
<td>ns</td>
</tr>
<tr>
<td>Pasture</td>
<td>8.44</td>
<td>8.32</td>
<td>0.69</td>
<td>ns</td>
</tr>
<tr>
<td>Total DMI</td>
<td>18.9</td>
<td>18.1</td>
<td>0.40</td>
<td>0.08</td>
</tr>
<tr>
<td>CP</td>
<td>3.18</td>
<td>2.91</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>7.69</td>
<td>7.31</td>
<td>0.21</td>
<td>ns</td>
</tr>
<tr>
<td>ADF</td>
<td>5.30</td>
<td>4.72</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>lignin</td>
<td>0.66</td>
<td>0.51</td>
<td>0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>starch</td>
<td>2.98</td>
<td>2.86</td>
<td>0.02</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

\(^a\) Cows fed TMR, total mixed ration (60:40 forage: concentrate) and mixed cool-season grasses (n = 8, CON). \(^b\) Cows fed TMR and mixed cool-season grasses plus annual forages (n = 8, AF). \(^c\) ns, non-significant, Significance declared at \(P < 0.05\). Trends at \(0.05 < P < 0.10\). DMI, dry matter intake. \(^d\) AF mixture included barley, wheat, rye, triticale, and hairy vetch.
Table 5-2 Fatty acid compositions of total mixed ration and pasture offered to lactating Jersey cows

<table>
<thead>
<tr>
<th>Fatty acid (g FA/100g)</th>
<th>TMR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CON&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AF&lt;sup&gt;c&lt;/sup&gt;</th>
<th>AF&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>18.8</td>
<td>17.7</td>
<td>19.6</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>2.8</td>
<td>1.8</td>
<td>1.9</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>18:1&lt;sup&gt;c&lt;/sup&gt; c9</td>
<td>13.3</td>
<td>10.8</td>
<td>5.4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>18:2&lt;sup&gt;c&lt;/sup&gt; c9,c12 (n-6)</td>
<td>31.8</td>
<td>35.0</td>
<td>21.3</td>
<td>47.8</td>
<td></td>
</tr>
<tr>
<td>18:3&lt;sup&gt;c&lt;/sup&gt; c9,c12,c15 (n-3)</td>
<td>27.4</td>
<td>28.7</td>
<td>43.8</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>ΣSFA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.0</td>
<td>22.8</td>
<td>25.7</td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td>ΣMUFA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>14.3</td>
<td>11.8</td>
<td>6.5</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>ΣPUFA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>60.6</td>
<td>65.2</td>
<td>67.7</td>
<td>66.6</td>
<td></td>
</tr>
<tr>
<td>Σn-6&lt;sup&gt;h&lt;/sup&gt;</td>
<td>31.9</td>
<td>35.2</td>
<td>21.5</td>
<td>48.6</td>
<td></td>
</tr>
<tr>
<td>Σn-3&lt;sup&gt;i&lt;/sup&gt;</td>
<td>28.7</td>
<td>30.1</td>
<td>46.2</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>1.1</td>
<td>1.2</td>
<td>0.5</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

Total fatty acids (% of DM) 3.3 2.5 2.4 1.4

<sup>a</sup>TMR= total mixed ration (57% of total diet), <sup>b</sup>Cows fed TMR, total mixed ration (60:40 forage: concentrate) and mixed cool-season grasses (n = 8, CON), <sup>c</sup>Cows fed TMR and mixed cool-season grasses plus annual forages (n = 8, AF), <sup>d</sup>AF mixture contained annual forages rye, wheat, barley, triticale, hairy vetch, <sup>e</sup>ΣSFA, sum of saturated fatty acids (12:0-24:0), <sup>f</sup>ΣMUFA, sum of monounsaturated fatty acids (16:1<sup>c</sup> c9 + 18:1<sup>c</sup> c9 + 18:1<sup>c</sup> c11 + 22:1<sup>c</sup> c9), <sup>g</sup>ΣPUFA, sum of polyunsaturated fatty acids (18:2<sup>c</sup> c9,c12 + 18:3<sup>c</sup> c9,c12,c15 + 20:2<sup>c</sup> c11,c14 + 20:3<sup>c</sup> c5,c8,c11 + 20:3<sup>c</sup> c11,c14,c17), <sup>h</sup>Σn-6, 18:2<sup>c</sup> c9,c12 + 20:2<sup>c</sup> c11,c14 + 20:3<sup>c</sup> c5,c8,c11, <sup>i</sup>Σn-3, 18:3<sup>c</sup> c9,c12,c15 + 20:3<sup>c</sup> c11,c14,c17.
Table 5-3 Estimated fatty acid intakes of total mixed ration and pasture offered to lactating Jersey cows

<table>
<thead>
<tr>
<th>Fatty acid Intake (g FA/d)</th>
<th>CON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SE</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>103</td>
<td>101</td>
<td>1.4</td>
<td>ns</td>
</tr>
<tr>
<td>18:0</td>
<td>13.6</td>
<td>13.0</td>
<td>0.2</td>
<td>ns</td>
</tr>
<tr>
<td>18:1 c9</td>
<td>69.3</td>
<td>56.2</td>
<td>0.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>18:2 c9,c12 (n-6)</td>
<td>185</td>
<td>149</td>
<td>2.5</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>18:3 c9,c12,c15 (n-3)</td>
<td>156</td>
<td>174</td>
<td>2.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>ΣSFA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>135</td>
<td>134</td>
<td>1.9</td>
<td>ns</td>
</tr>
<tr>
<td>ΣMUFA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>76.1</td>
<td>63.3</td>
<td>0.9</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>ΣPUFA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>349</td>
<td>333</td>
<td>5.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Σn-6&lt;sup&gt;g&lt;/sup&gt;/Σn-3&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1.1</td>
<td>0.8</td>
<td>&lt; 0.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>total fatty acids</td>
<td>560</td>
<td>529</td>
<td>7.9</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cows fed a TMR, total mixed ration, (60:40 forage: concentrate) and mixed cool-season grasses (n = 8, CON). <sup>b</sup>Cows fed TMR and mixed cool-season grasses plus annual forages wheat, barley, rye, triticale, hairy vetch (n = 8, AF). <sup>c</sup>non-significant (ns), Significance declared at <i>P</i> < 0.05. <sup>d</sup>ΣSFA, sum of saturated fatty acids (12:0-24:0), <sup>e</sup>ΣMUFA, sum of monounsaturated fatty acids (16:1 <i>c</i>9 + 18:1 <i>c</i>9 + 18:1 <i>c</i>11 + 22:1 <i>c</i>9), <sup>f</sup>ΣPUFA, sum of polyunsaturated fatty acids (18:2 <i>c</i>9, <i>c</i>12+ 18:3 <i>c</i>9, <i>c</i>12,<i>c</i>15 + 20:2 <i>c</i>11,<i>c</i>14 + 20:3 <i>c</i>5,<i>c</i>8,<i>c</i>11 + 20:3 <i>c</i>11,<i>c</i>14,<i>c</i>17), <sup>g</sup>Σn-6, 18:2 <i>c</i>9, <i>c</i>12 + 20:2 <i>c</i>11,<i>c</i>14 + 20:3 <i>c</i>5,<i>c</i>8,<i>c</i>11, <sup>h</sup>Σn-3, 18:3 <i>c</i>9, <i>c</i>12,<i>c</i>15 + 20:3 <i>c</i>11,<i>c</i>14,<i>c</i>17.
<table>
<thead>
<tr>
<th>Fatty Acid (g/100g FA)</th>
<th>Bacteria</th>
<th></th>
<th></th>
<th></th>
<th>Protozoa</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CONa</td>
<td>AFb</td>
<td>SE</td>
<td>P-valuec</td>
<td>CON</td>
<td>AF</td>
<td>SE</td>
<td>P-value</td>
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<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-13:0</td>
<td>0.31</td>
<td>0.29</td>
<td>0.02</td>
<td>ns</td>
<td>0.08</td>
<td>0.08</td>
<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>13:0</td>
<td>0.21</td>
<td>0.19</td>
<td>0.02</td>
<td>ns</td>
<td>0.14</td>
<td>0.12</td>
<td>0.03</td>
<td>ns</td>
</tr>
<tr>
<td>iso-14:0</td>
<td>0.81</td>
<td>0.81</td>
<td>0.08</td>
<td>ns</td>
<td>0.42</td>
<td>0.47</td>
<td>0.07</td>
<td>ns</td>
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<tr>
<td>iso-15:0</td>
<td>1.37</td>
<td>1.39</td>
<td>0.08</td>
<td>ns</td>
<td>0.32</td>
<td>0.35</td>
<td>0.03</td>
<td>ns</td>
</tr>
<tr>
<td>anteiso-15:0</td>
<td>3.15</td>
<td>3.07</td>
<td>0.29</td>
<td>ns</td>
<td>1.15</td>
<td>1.03</td>
<td>0.08</td>
<td>ns</td>
</tr>
<tr>
<td>15:0</td>
<td>2.42</td>
<td>2.20</td>
<td>0.25</td>
<td>ns</td>
<td>1.19</td>
<td>1.19</td>
<td>0.08</td>
<td>ns</td>
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<td>iso-16:0</td>
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<td>1.38</td>
<td>1.23</td>
<td>0.07</td>
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<tr>
<td>16:0</td>
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<td>18.5</td>
<td>0.49</td>
<td>ns</td>
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<td>29.2</td>
<td>2.57</td>
<td>ns</td>
</tr>
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<td>iso-17:0</td>
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<td>0.41</td>
<td>0.02</td>
<td>ns</td>
<td>0.28</td>
<td>0.20</td>
<td>0.04</td>
<td>ns</td>
</tr>
<tr>
<td>anteiso-17:0</td>
<td>0.20</td>
<td>0.18</td>
<td>0.01</td>
<td>ns</td>
<td>0.15</td>
<td>0.16</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>17:0</td>
<td>0.68</td>
<td>0.76</td>
<td>0.02</td>
<td>ns</td>
<td>0.37</td>
<td>0.37</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>18:0</td>
<td>44.7</td>
<td>46.2</td>
<td>1.41</td>
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* Cows fed total mixed ration (TMR, 60:40 forage: concentrate) and mixed grasses (n = 5, CON). * Cows fed TMR and mixed cool-season grasses with 7.3% dry matter intake as annual forages wheat, rye, barley, triticale, and hairy vetch (n = 5, AF). * non-significant (ns), Significance declared at $P < 0.05$, Trends at 0.05 < $P < 0.10$, 4 ΣSFA, sum of saturated fatty acids (9:0-24:0); 5 ΣOCFA, sum of odd-chain fatty acids (5:0-23:0); 6 ΣBCFA, sum of branched-chain fatty acids (iso-13:0 + iso-14:0 + iso-15:0 + anteiso-15:0 + iso-16:0 + iso-17:0 + anteiso-17:0); 7 ΣMUFA, sum of monounsaturated fatty acids (16:1-18:1); 8 ΣCLA, sum of conjugated linoleic acids.
Table 5-5 Milk content and serving size of selected fatty acids from lactating Jersey cows offered traditional pasture or annual forages plus traditional pasture

<table>
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<th>AF$^b$</th>
<th>SE</th>
<th>P-value$^c$</th>
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<th>AF</th>
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Cows fed TMR, total mixed ration (60:40 forage: concentrate) and mixed grasses (n = 8, CON), Cows fed TMR and mixed grasses with 7.3% dry matter intake annual forages, wheat, rye, barley, triticale, and hairy vetch (n = 8, AF), ns, non-significant, Significance declared at P < 0.05, Trends at 0.5 < P < 0.10, ∑SFA, sum of saturated fatty acids (4:0-24:0); ∑OFA, sum of odd-chain fatty acids (5:0-23:0); ∑BCFA, sum of branched-chain fatty acids (iso-13:0 + anteiso-13:0 + iso-14:0 + iso-15:0 + anteiso-15:0 + iso-16:0 + iso-17:0 + anteiso-17:0 + iso-18:0); ∑MUFA, sum of monounsaturated fatty acids (14:1-22:1); ∑PUFA, sum of polyunsaturated fatty acids (18:2-22:5); ΣCLA, sum of conjugated linoleic acids, Σn-6, 18:3 c9, c12, c15 + 20:5 c5, c8, c11, c14, c17 + 22:5 c7, c10, c13, c16, c19, Σn-3, 18:2 c9, c12 + 18:3 c6, c9, c12 + 20:2 c11, c14 + 20:3 c5, c8, c11 + 20:4 c5, c8, c11, c14, preformed, > 18 carbons, mixed, 16:0 and 16:0 c9, de novo, < 16 carbons and includes 17:0.
Figure 5-1 Distribution of rumen bacterial 16S and protozoal 18S rRNA genes to nearest valid taxa from lactating Jersey cows offered cool-season grass pasture or cool-season grasses plus annual forages

(A) Relative abundances of protozoal genera by targeting the V3-V4 region of the 18S rRNA gene, (B) Relative abundances of bacterial genera belonging to the phylum Firmicutes by targeting the V1-V3 region of the 16S rRNA gene, (C) Relative abundances of bacterial genera belonging to the phylum Bacteroidetes by targeting the V1-V3 region of the 16S rRNA gene, CON, control, Cows fed TMR, total mixed ration (60:40 forage: concentrate) and mixed grasses (n = 8), AF, annual forage, Cows fed TMR and mixed grasses plus annual forages, wheat, rye, barley, triticale, and hairy vetch (n = 8), Significance declared at *P < 0.05, Trends at †0.5 < P < 0.10, g, genus, f, family, o, order.
Supplementary Table 5-6: Milk content of select fatty acids from lactating Jersey cows consuming mixed grass/legume pasture or annual forage crops plus mixed grass/legume pasture

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<th>AF</th>
<th>SE</th>
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<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>Σ18:1 t</td>
<td></td>
<td>20.55</td>
<td>18.32</td>
<td>0.46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ΣMUFAd</td>
<td></td>
<td>22.31</td>
<td>20.92</td>
<td>0.31</td>
<td>0.02</td>
</tr>
<tr>
<td>ΣPUFAe</td>
<td></td>
<td>3.60</td>
<td>3.55</td>
<td>0.07</td>
<td>ns</td>
</tr>
<tr>
<td>ΣCLAe</td>
<td></td>
<td>0.60</td>
<td>0.61</td>
<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>Σn-6i</td>
<td></td>
<td>0.70</td>
<td>0.66</td>
<td>0.02</td>
<td>ns</td>
</tr>
<tr>
<td>Σn-3i</td>
<td></td>
<td>1.93</td>
<td>1.86</td>
<td>0.04</td>
<td>ns</td>
</tr>
</tbody>
</table>
Least-squares means are based on n=8 CON and n=8 alternative forage-fed, AF lactating cows, significance declared at \( P < 0.05 \) and trends at \( 0.05 < P < 0.10 \).\(^a\) ns, non-significant \( \Sigma \) SFA, sum of saturated fatty acids (4:0-24:0); \( \Sigma \) OCFA, sum of odd-chain fatty acids (5:0-23:0); \( \Sigma \) BCFA, sum of branched-chain fatty acids (iso-13:0 + anteiso-13:0 + iso-14:0 + iso-15:0 + anteiso-15:0 + iso-16:0 + iso-17:0 + anteiso-17:0 + iso-18:0); \( \Sigma \) MUFA, sum of monounsaturated fatty acids (14:1-22:1); \( \Sigma \) PUFA, sum of polyunsaturated fatty acids (18:2-22:5); \( \Sigma \) CLA, sum of conjugated linoleic acids, \( \Sigma \) n-3, 18:2 c9, c12 + 18:3 c6, c9, c12 + 20:2 c11, c14 + 20:3 c5, c8, c11 + 20:4 c5, c8, c11, c14, \( \Sigma \) n-6, 18:2 c9, c12 + 20:2 c11, c14 + 20:3 c5, c8, c11, c15, + 20:5 c5, c8, c11, c14, + 22:5 c7, c10, c13, c16, c19.
Supplementary Table 5-7: Proportions of rumen volatile fatty acids from lactating Jersey cows consuming cool-season grass/legume pasture or annual forages plus cool-season grass/legume pasture

<table>
<thead>
<tr>
<th>Volatile fatty acid (%)</th>
<th>CON(^a)</th>
<th>AF(^b)</th>
<th>SE</th>
<th>P-value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td>69.19</td>
<td>69.79</td>
<td>0.68</td>
<td>ns</td>
</tr>
<tr>
<td>propionate</td>
<td>16.57</td>
<td>16.38</td>
<td>0.42</td>
<td>ns</td>
</tr>
<tr>
<td>butyrate</td>
<td>11.63</td>
<td>11.34</td>
<td>0.41</td>
<td>ns</td>
</tr>
<tr>
<td>isobutyrate</td>
<td>0.94</td>
<td>0.93</td>
<td>0.03</td>
<td>ns</td>
</tr>
<tr>
<td>valerate</td>
<td>1.02</td>
<td>0.95</td>
<td>0.09</td>
<td>ns</td>
</tr>
<tr>
<td>isovalerate</td>
<td>0.55</td>
<td>0.69</td>
<td>0.14</td>
<td>ns</td>
</tr>
<tr>
<td>acetate:propionate</td>
<td>4.18</td>
<td>4.27</td>
<td>0.14</td>
<td>ns</td>
</tr>
<tr>
<td>Total VFA(^d) (mM)</td>
<td>77.91</td>
<td>76.98</td>
<td>6.04</td>
<td>ns</td>
</tr>
</tbody>
</table>

\(^a\) control, CON (n = 8) cows consuming total mixed ration plus cool-season grass/legume pasture, \(^b\) annual forage, AF cows (n = 8) fed total mixed ration plus cool-season grass/legume pasture, wheat, rye, barley, triticale, and hairy vetch, \(^c\) ns, non-significant, significance declared at \(P < 0.05\), \(^d\) VFA, volatile fatty acids
Chapter 6. Summer Annual Forages Offered To Mid-Lactation Jersey Cows
Altered Milk Fatty Acid Contents Without Changing Key Rumen Microbial Fatty Acids And Taxa

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Keywords: bacteria, buckwheat, oat, dairy, organic, protozoa
6.1 Abstract

In the Northeast U.S., there is interest in feeding annual forages (AF), such as cereal and broadleaf forages, to dairy cows when cool-season perennial grass growth is decreased during the summer. The purpose of the study was to determine if rumen microbial community structures and fatty acid (FA) and milk FA contents, differed between cows offered cool-season grass-legume pasture (control group, CON) or cool-season grass-legume pasture 30% strip-tilled with AF (AF group, buckwheat, chickling vetch, oat). A 21d experiment was conducted with 16 Jersey cows (n = 8 CON, n= 8 AF). AF-fed cows had lower dry matter intakes (DMI) of starch on pasture than CON-fed cows. AF were an estimated 5.7% of the total DMI. Dietary FA intakes did not differ between AF and CON-fed cows. The AF buckwheat and chickling vetch contained lower NDF contents in comparison to cool-season grasses. Protozoal and bacterial cell proportions of PUFA and rumen biohydrogenation intermediates (e.g., 18:1 trans isomers) and abundances of major protozoal (e.g., Entodonium) and bacterial taxa (e.g., Prevotella) did not differ between treatment groups. Milk de novo FA contents (g FA/kg milk) were greater in AF than CON-fed cows, but did not differ by serving whole milk. Contents of palmitic acid (16:0) per serving were greater from AF than CON cows. Our findings suggest that milk FA contents were altered through an alternative feeding strategy without impacting major rumen microbial cell membrane FA and taxa.

6.2 Introduction
Dairy products are key sources of nutrients, such as proteins, vitamins, minerals, and fats. Cows’ milk contains 3 to 5% fat with more than 400 different fatty acids (FA) identified\(^1\) and the milk fat contains several unique bioactive FA that impact human health\(^2\). Polyunsaturated FA (PUFA) are precursors of signaling molecules\(^3\) and include the essential FA linoleic (LA, 18:2 \(c_9,c_{12}\)) and \(\alpha\)-linolenic (ALA, 18:3 \(c_9,c_{12},c_{15}\)) acids. Branched- (BCFA) and odd-chain FA (OCFA), mainly derived from microbial cells, are associated with apoptosis of cancer cells\(^4,5\) and greater cell membrane fluidity\(^6\), respectively. Diet is a key determinant of rumen-derived FA and hence, the FA transported to the mammary gland and incorporated into milk. Less is known about the FA derived from microbial cells and their contributions to milk fat.

It is important to understand how the milk content can be altered by the animals’ diet in concert with the ruminal microbiota and their respective FA metabolism products. Ruminal microorganisms ferment carbohydrates into volatile FA (VFA), the main energy sources for milk production. Bacteria represent 90% of the total ruminal microbes and are the most diverse population in the rumen\(^7\). Although less numerous than ruminal bacteria (\(10^{10}-10^{11}\) cells/mL rumen fluid)\(^8\), protozoa have been suggested to contribute to half of the rumen microbial biomass (\(10^4-10^6\) cells/mL rumen fluid)\(^9\). Dietary PUFA, including ALA and LA are bacteriostatic\(^10\). Some bacteria, such as, *Butyrivibrio fibrisolvens*, overcome this problem by biohydrogenating PUFA into stearic acid (SA, 18:0) and forming 18:1 \(\text{trans}\) isomers and conjugated linoleic acid (CLA) isomers as intermediates. Ruminal bacteria synthesize BCFA and OCFA for incorporation into their cell membranes\(^11\). Protozoal cells contain less BCFA and OCFA\(^12\), and unlike bacteria, engulf chloroplasts rich in PUFA\(^13\). Once microbial cells leave the rumen and
subsequently enter the duodenum, their FA are absorbed and later contribute to the milk FA composition. Therefore, characterizing the bacterial and protozoal FA compositions and their community structures will provide novel insight into the relationship between rumen microbial FA, microbiota, and milk FA.

In the Northeastern U.S., decreased growth of cool-season grasses occur during the mid to late summer. As organic dairy farmers are required to graze their cows for at least 120 days a year\(^{14}\) and interested in extending the grazing season\(^{15}\), forage production is of paramount importance. One practical approach to extend the grazing season, while potentially producing more forage, is to incorporate annual forages (AF) \((e.g.,\) buckwheat) into traditional legume-grass pasture mixtures. Liu et al.\(^{16}\) displayed that oat has a greater total 18:1 content than wheat. Buckwheat consumption provided greater milk fat concentrations of PUFA in comparison to ryegrass\(^{17}\). No studies, however, have demonstrated whether or not the consumption of an AF mixture influences rumen microbial taxa and their FA profiles, or milk FA contents in dairy cows under organic farming practices. We hypothesized that the FA composition of the AF diet will have a greater proportion of PUFA and demonstrate greater nutrient quality than the CON diet, thus, altering the relative abundances of rumen bacterial and protozoal taxa, microbial bioactive FA compositions, and milk content of bioactive FA per serving of whole milk.

The main objective of the study was to determine if a traditional legume-grass pasture 30% strip-tilled with AF, would alter rumen microbial and milk FA compositions in Jersey cows. Our specific objectives were to (1) quantify dietary FA, (2) quantify microbial cell FA compositions, (3) characterize rumen microbial community structures,
(4) determine the milk FA content, and (5) quantify contents of FA per serving of whole milk from Jersey cows that consumed a CON pasture of legumes and mixed cool-season grasses with or without AF.

6.3 Methods and Materials

6.3.1 Study Design

The University of New Hampshire’s Institutional Animal Care and Use Committee (IACUC) approved all animal sampling methods under protocol 150302. A 21d experiment was performed between July-August 2015. Sixteen lactating Jersey cows were co-housed as described by Resende et al. at the University of New Hampshire Burley-Demeritt Organic Research Farm\(^{18}\). Eight cows consuming TMR plus cool-season grass/legume pasture were matched by days in milk (143 ± 58 DIM), parity (1.4 ± 53 lactations), and milk yield (18.1 ± 3.9 kg/d) to eight cows consuming TMR plus cool-season grasses and legumes with an AF mixture 30% strip-tilled. Each experimental period consisted of a 14d adaptation period. All covariate period samples were collected on days 3 to 4 prior to the 21d experimental period. During the covariate period, all 16 cows consumed the same diet (TMR and CON pasture). Forage (d18-21), rumen digesta (d20-21), and milk samples (d19-21) were collected after the adaptation period.

6.3.2 Diet

Cows were fed TMR (60:40, forage:concentrate) formulated to 60% of the total daily DMI after the AM milking (0800 h). Cows were trained to use the Calan door system (American Calan Inc., Northwood, NH), to measure the individual DMI. Cows were offered pasture formulated to meet 40% of the DMI after the PM milking (1600 h) until the following AM milking at (0600 h). Chromium oxide was fed to cows as an
external biomarker to estimate DMI of pasture. Cows consumed 1 kg of pelleted concentrate with chromium oxide (6.23 g/d) for 10 consecutive days during AM and PM feedings of TMR. Individual fecal samples were collected twice daily for five consecutive days (d17-d21). Pasture DMI was calculated using previously established methods\(^{(19)}\).

Forage in the TMR comprised of 100% mixed grass-legume baleage, while concentrate contained: organic corn grain ground (55.0%), wheat middlings (18.3%), soybean meal (7.6%), cane molasses (3.4%), salt (3.0%), canola meal (2.5%), ground barley (6.9%), calcium carbonate (1.8%), magnesium oxide (0.6%), Diamond V XP\(^{TM}\) (0.5%), Green Mountain Feeds Nuplex\(^{TM}\) (0.1%), magnesium sulfate (0.1%), and sodium bicarbonate (0.1%). The CON pasture contained orchardgrass (\textit{Dactylis glomerata}), timothy grass (\textit{Phleum pratense}), and Kentucky bluegrass (\textit{Poa pratensis}), and white clover (\textit{Trifolium repens}). The AF pasture contained a 30% strip-tilled AF mixture within the grass-legume pasture. The AF mixture included oat (\textit{Avena sativa}), buckwheat (\textit{Fagopyrum esculentum}), chickling vetch (\textit{Lathyrus sativus}), millet (\textit{Pennisetum glaucum}), and teff (\textit{Eragrostis tef}). Note that millet and teff were not observed on pasture, but matured as monocultures at the UNH horticulture farm.

Pasture (pre-grazed) and TMR samples were collected for four consecutive days (d18-d21) and immediately stored at -20°C until further analysis. TMR samples were composited by period and Dairy One (Ithaca, NY) provided the nutrient analyses. Sixty pre-grazing samples were collected from CON and AF paddocks. Immediately after collection, samples were thoroughly mixed. A quarter of the sample was used for botanical composition and nutrient analyses of individual components with Cumberland
Valley Analytical Services (Hagerstown, MD), while another quarter was used for DM calculations. The samples collected for CON pasture botanical composition were divided into grasses, legumes, broadleaf weeds, and dead material. The AF pasture samples collected for botanical compositions were divided into grasses, AF broadleaf (buckwheat plus chickling vetch), oats, broadleaf weeds (Plantago major), and dead material. Feedstuff samples collected for nutrient and botanical compositions were dried in a forced-air oven at 65°C for 48 h to determine percent DM. Methods described by Bainbridge et al. (20) were used for forage FA analyses of TMR, pasture and individual botanical compositions.

6.3.3 Milk Sample Collection and FA
On d19-21, milk samples (100 mL) were collected for four consecutive milkings. Milk sample aliquots (50 mL) for milk solids analyses were preserved in 2-bromo-2-nitropropan-1,3-diol, pooled by cow, and analyzed by the method described by Resende et al. (18). Animal performance measurements included milk fat % and yields and milk production. A 50 mL aliquot for FA analyses was collected and stored at -20°C without preservative until further analysis. Samples for lipid extraction were thawed, composited by individual cow and milk production, spun at 17,800 x g at 8°C for 30 min and the cream layer was collected and frozen at -20°C until further FA analysis. Milk lipid extraction and FA analysis using gas-liquid chromatography (GLC) were performed as described by Bainbridge et al. (20). Both milk content (g FA/kg milk) and amount of FA provided per serving (244g) of 3.25% fat whole milk were calculated as described by Bainbridge et al. (21). The milk FA content is reported as g FA/kg milk to reflect animal performance (milk production and fat yield). Where possible, milk FA contents (g/100g
FA or g/kg FA) from previously published studies were converted to g FA/kg milk for use in the discussion section.

**6.3.4 Whole Rumen Digesta Collection**

During the covariate period and the last two days of each experimental period, 500 mL of individual whole rumen digesta samples were collected by esophageal intubation after AM milking. Rumen digesta collection occurred on two consecutive days (-4d and -3d, covariate; d20-21, experimental) with four CON and four AF cows randomly sampled each day. Immediately after sample collection, saliva was poured off and 1.5 mL of 1% methyl cellulose was added to enable microbial detachment from forage particles. Approximately 420 mL of whole rumen digesta were used for microbial fractionation and 50 mL for VFA analyses. Samples collected for VFA analyses were strained through a layer of cheese cloth, 50% (vol/vol) H₂SO₄ was added, and samples were frozen at -20°C. VFA analyses using GLC were performed by West Virginia University Rumen Fermentation Profiling Laboratory (Morgantown, WV) (Supplementary 6-6).

**6.3.5 Microbial Diversity and Density**

Microbial DNA was extracted from 250 µL whole rumen digesta (i.e., fluid and solids) with the repeated-bead beating plus column method described by Yu and Morrison(22). Real-time PCR determined the microbial densities from each individual sample in triplicate as previously described(23,24) (Supplementary Table 6-7). In order to classify sequences to microbial taxa and measure diversity, the bacterial primer pair 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) (25) and 519R (5’-GWATTACCGCGGCKGCTG-3’) (26) amplified the V1-V3 hypervariable regions of the 16S rRNA gene,
while the protozoal-specific primer P-SSU-316F (5’-GCTTTCGWTGTTAGTGATT-3’) (27) and GIC758R (5’-CAACTGTCTCTATKAAYCG-3’) (28) amplified the V3-V4 hypervariable regions of the 18S rRNA gene. Bacterial DNA extract was diluted to 10 ng/µL and protozoal DNA was not. For each bacterial PCR, 2 µL of DNA and 31.5 µL of double-distilled water (ddH₂O), 2.5 µL 27F, 2.5 µL of 519R, 1 µL of deoxynucleotide (dNTP), 10 µL of HF buffer (New England BioLabs, Ipswich, MA), and 0.5 µL of Phusion DNA polymerase (New England BioLabs). The GeneAmp PCR Systems 9700 (Life Technologies, Carlsbad, CA) thermal cycler conditions to amplify the bacterial 16S rRNA gene included: a hot start (98 °C, 4 min), 35 cycles of denaturation (98 °C, 10s), annealing (56°C, 30s), extension (72 ºC, 30s). Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, CA) settings to amplify the protozoal 18S rRNA gene included: a hot start (94 °C, 240s), 35 cycles of denaturation (94 °C, 30s), annealing (55 ºC, 30s), and extension (72 ºC, 60 s). For both protocols, the last cycle included a final extension at 72 ºC for 6 min. Positive (DNA from bacterial and protozoal pellets), and negative (double-distilled water) controls were run with all reactions. DNA amplicons (15-20 ng/µL) were purified with the Qiagen QIAquick Gel Extraction Kit (Hilden, Germany) and submitted to Molecular Research Laboratories (Shallowater, TX) for paired-end sequencing with the Illumina MiSeq (v.3) sequencing platform.

6.3.6 Bioinformatic Analyses Of Microbial Sequence Reads
MOTHUR v. 1.33 (29) was used for all in-house bioinformatic analyses. All 16S and 18S rRNA gene sequence reads were analyzed using a previously described workflow (23). Briefly, the command, trim.seqs, removed the barcodes from the amplicons and set a minimum average quality score of 25. A Needleman-Wunsch pairwise
alignment method aligned bacterial and protozoal data sets. Reference alignment files of known protozoal 18S rRNA gene sequence reads were used. The bacterial reference alignment file contained 500 rumen bacterial 16S rRNA gene sequence reads from the unaligned data set. The conserved regions of the 500 bacterial 16S rRNA gene sequence reads were aligned with in-house Perl Scripts previously used by Bainbridge et al. \cite{24} Alignments were manually checked for any misaligned sequences. UChime \cite{30} chimera checked all sequences. Methods to classify microbial taxa, determine alpha diversity, and operational taxonomic units (OTU) were previously reported by \cite{24,31}. Subsamples of 5,000 16S rRNA gene and 10,000 18S rRNA gene sequence reads per individual, determined by computing power, were used to calculate pairwise distances with cutoff parameters at 0.05 and 0.04 for bacteria and protozoa, respectively. Distance matrices were used to cluster sequences into OTU and measure community diversity.

### 6.3.7 Bacterial and Protozoal FA Analyses

Individual bacterial and protozoal fractions were obtained by the differential centrifugation procedure described by Or-Rashid et al. \cite{12} and with modifications outlined by Cersosimo et al. \cite{23}. After repeating the protozoal pellet cleanup step four times, the sample was viewed under a microscope to observe possible forage contamination. An acceptable sample contained < 5% plant matter. Individual bacterial and protozoal pellets were stored at -80°C to stop all microbial activity. Pellets were lyophilized in a freeze-dryer (Labconco, Kansas City, MO) for 48h. Subsequently, bacterial and protozoal lipids were extracted, the FA methyl esters were produced by transesterification \cite{32} and analyzed via GLC using the methods described by Cersosimo et al. \cite{23}. Total bacterial and protozoal FA compositions were determined as described by Bainbridge et al. \cite{20}
with the exception of the oven temperature program. Briefly, the program included an initial temperature of 45°C held for 4 min, then programmed to increase by 13°C/min to 167°C with a 40 min hold, and programmed to increase by 4°C/min to 218°C with a 23 min hold at the end. GC-MS analyses described by Cersosimo et al.\textsuperscript{(23)} identified microbial dimethyl acetals.

**6.3.8 Statistical Analyses**

A completely randomized block design with the MIXED procedure in SAS tested the effect of treatment between CON and AF cows after 21d. The statistical model included the covariate period. Cows were blocked by DIM, parity, and milk production. Pearson correlations, using the CORR procedure in SAS, determined the relationships between microbial taxa and their cellular FA and with milk fat contents. The corrplot package in RStudio was used to visualize the Pearson’s correlations. All data are reported as the least-squares means ± the standard error mean (SE) with statistical significance declared at $P < 0.05$ and trends at $0.05 \leq P \leq 0.10$.

**6.4 Results**

**6.4.1 Dietary Nutrients And Fatty Acid Contents**

On a DM-basis, the botanical composition of CON pasture contained 69.0% mixed grasses, 11.0% legumes, and 20.0% weeds/dead material: the AF pasture contained 61.0% mixed grasses, 13.0% legumes, 9.0% weeds/dead material, 14% AF broadleaf, and 1% oats. Proportions of individual FA did not differ between treatment groups (Table 6-1). Chickling vetch and buckwheat were in their reproductive (flowering) stages, with numerically lower NDF (35.2% DM), ALA (4.7 mg/g FA), and LA (4.1 mg/g) contents compared to the AF grasses (55.3% NDF, 11.8 mg/g FA, 4.4
mg/g FA, respectively). AF cows had greater DMI of lignin than CON cows (CON: 0.71 kg/d, AF: 1.17 kg/d) and tended to have greater DMI of starch than CON-fed cows (CON: 2.72 kg/d, AF: 2.93 kg/d, \(P = 0.07\), Table 6-2). Total intakes of total and individual FA did not differ between treatment groups (Table 6-2). DMI of TMR did not differ between treatment groups (Table 6-2) and was 57% of the total DMI.

### 6.4.2 Rumen Microbial Fatty Acid Compositions

Bacterial cell contents of SA and PA did not vary between treatment groups (Table 6-3). Vaccenic acid (18:1 t11) and oleic acid (18:1 c9) were the most abundant UFA identified in the bacterial cells. Contents of total bacterial trans-18:1 tended to be lower in the AF group (5.32 g/100g FA) than in the CON groups (5.90 g/100g FA, \(P = 0.08\)), while the content of total BCFA and total OCFA did not differ between groups (Table 6-3). No differences in protozoal cell FA were observed between treatment groups. The most abundant FA identified in protozoal cells were PA and SA. Contents of protozoal cell LA and ALA were numerically greater than from bacterial cells (Table 6-3).

### 6.4.3 Rumen Microbial Taxa

Raw protozoal 18S and bacterial 16S rRNA gene sequences reads are accessible through the Sequence Read Archive of the National Center for Biotechnology Information, under the accession number SRP081268.

All protozoa belonged to the phylum Ciliophora and the majority of the rumen protozoa belonged to the order Entodiniomorphida (97.8%) and the family Ophryoscolecidae (97.3%). The less abundant protozoal genus (< 1% of total protozoa)
from the order Entodiniomorphida included *Ophyrosclex* (0.5%). The protozoal genera, *Dasytricha* and *Isotricha* were the only genera belonging to the order Vestibuliferida (2.2%). Differences in the relative abundances of protozoal genera (Figure 6-1), diversity, and density between CON and AF-fed cows were not observed (Supplementary Table 6-6). Relative abundances of the genus *Metadinium* tended to be greater in AF-fed cows than CON-fed cows (*P* = 0.07, Figure 6-1A).

The majority of rumen bacterial 16S rRNA gene sequences belonged to the phyla Firmicutes (51.2%) and Bacteroidetes (45.2%). Prevalent bacterial classes included Bacteroidia (43.3%) and Clostridia (49.8%), bacterial classes less than 1% abundance included Flavobacteria, Sphingobacteria, Bacilli, Mollicutes, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria. Rumen bacteria of the orders Bacteroidales (43.3%) and Clostridiales (49.8%) were prevalent amongst all samples, while bacteria belonging to the orders Aquificales, Flavobacteriales, Sphingobacteriales, Bacillales, Lactobacillales, Mycoplasmatales, Burkholderiales were each less than 1% of the total bacteria.

Relative abundances of the prevalent phyla Firmicutes, Bacteroidetes, and Proteobacteria did not differ between CON-fed and AF-fed cows. Differences in the more abundant bacterial families Ruminococcaceae, Lachnospiraceae, and Prevotellaceae were not observed between treatment groups. The bacterial family Succinivibrionaceae was more abundant in AF-fed cows (0.19%) than in CON-fed cows (0.06%) (*P* = 0.04). AF-fed cows (0.62%) had more unclassified bacterial families than CON-fed cows (0.43%) (*P* = 0.04). Bacteria belonging to the genus *Coprococcus* were more abundant in AF-fed (1.96%) than in CON-fed (1.46%) cows (*P* = 0.04) (Figure 6-1B), while genera
belonging to the Bacteroidetes phylum did not differ between groups (Figure 6-1C). Densities and diversity measures did not differ between treatment groups (Supplementary Table 6-7).

6.4.4 Correlations Between Microbial Taxa And their FA

The most abundant bacterial genus *Prevotella* correlated with bacterial ALA (r = 0.37, \( P = 0.04 \)). *Butyrivibrio* tended to correlate with 18:1-trans (r = 0.30, \( P = 0.09 \)). *Lachnospira* was negatively correlated with bacterial *a iso*-17:0 (r = -0.38, \( P = 0.03 \)), and ALA (r = -0.49, \( P < 0.01 \)), and positively correlated with milk fat % (r = 0.35, \( P < 0.05 \)). *Coprococcus* (r = 0.39, \( P = 0.02 \)) and *Ruminococcus* (r = 0.37, \( P = 0.04 \)) were positively correlated, while unclassified members of the family Ruminococcaceae were negatively correlated (r = -0.45, \( P < 0.01 \)) with bacterial VA. Additional rumen bacterial taxa, including phyla and genera, did not relate to milk fat yield or %.

Protozoal genera *Entodinium* (r = 0.56, \( P < 0.01 \)) and *Diplodinium* (r = 0.40, \( P = 0.02 \)) were positively correlated, while *Epidinium* (r = -0.36, \( P = 0.04 \)) was negatively correlated with protozoal *a iso*-15:0 (Figure 6-2). *Eudiplodinium* was correlated with ALA (r = 0.36, \( P = 0.04 \)) and total PUFA (r = 0.38, \( P = 0.03 \)). *Epidinium* was positively correlated with protozoal OCFA (r = 0.63, \( P < 0.001 \)) and *Entodinium* with BCFA (r = 0.40, \( P = 0.04 \)) (Figure 6-2). Protozoa belonging to the genus *Diplodinium* correlated with milk fat yield (r = 0.58, \( P < 0.05 \)) and milk fat % (r = 0.30, \( P < 0.05 \)).

6.4.5 Milk Fatty Acid Content Per Kg Milk Produced And Serving Of Whole Milk

Milk contents of *de novo* (\( P < 0.01 \)) and mixed (\( P = 0.01 \)) FA were greater in the AF-fed cows (12.75 and 15.56 g/kg milk, respectively) than in CON-fed cows (11.73 and 13.66 g/kg milk, respectively) (Table 6-5). Milk content of PA was 15.73 g/kg milk from
AF-fed cows and 12.70 g/kg milk ($P = 0.02$) from CON-fed cows. Milk OCFA and SFA contents were greater from AF cows (0.96 g/kg milk, 32.75 g/kg milk, respectively) than from CON (0.86 g/kg milk, 30.01 g/kg milk, respectively) ($P < 0.05$). No differences in the milk contents of total MUFA, 18:1-\textit{trans}, n-3 FA, n-6 FA, or BCFA were observed between groups.

Cows consuming CON pasture tended to provided more \textit{aiso}-15:0 and \textit{iso}-17:0 per serving of whole milk ($P = 0.09$) than the AF-fed cows (Table 6-5). There was a trend ($P = 0.09$) for the content of LA per serving to be greater in milk from CON-fed cows (94 mg/serving) than from AF-fed cows (89 mg/serving). Content of PA per serving was greater in milk from AF-fed cows (2529 mg/serving) than from CON-fed cows (2378 mg/serving, $P = 0.02$). Content of SA per serving tended to be lower in milk from AF-fed cows (836 mg/serving) than in milk from CON-fed cows (944 mg/serving, $P = 0.05$). No differences in the content of LA, ALA, total CLA, n-3 FA, n-6 FA, \textit{trans}-18:1 isomers, and BCFA per serving were observed between groups (Table 6-5).

### 6.5 Discussion

Breed, lactation stage, parity, and diet, are all factors that influence rumen bacterial community structures$^{23,24,31,34}$. Although it is well understood that diet is a significant determinant of rumen bacterial taxa present, less is known about how diet influences their FA profiles. Bas \textit{et al.} demonstrated that dietary NDF related to variations observed in the amounts of bacterial cell OBCFA$^{35}$. A greater NDF content led to increased contents of cellulolytic bacteria, rich in BCFA$^{32}$. Cool-season grasses with similar NDF contents were the most abundant pasture components in CON and AF

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pastures. AF broadleaf had similar NDF contents (35.9% of DM) to the TMR (32.8% of DM), but the DMI of TMR was 57% of the total diet, which may explain the lack of differences in total bacterial cell OBCFA.

Variations observed in the bacterial cell OBCFA contents are thought to reflect changes in the abundance of certain bacterial populations. For example, anteiso-15:0 was increased in bacteria that ferment sugar and pectin (e.g., *Prevotella ruminicola*), whereas iso-BCFA in bacteria were predominantly found in cellulolytic bacterial populations (e.g., *Ruminococcus flavefaciens*)\(^{(36,37)}\). Bacterial anteiso-15:0 contents were not associated with relative abundances of *Prevotella*, however, protozoal anteiso-15:0 was positively correlated to the abundances of *Entodinium* and *Diplodinium*. Contents of total rumen bacterial iso-FA were greater than total anteiso-FA contents, suggesting a greater abundance of ruminal cellulolytic bacteria from cows consuming pasture. Contents of iso-17:0, an abundant BCFA typically observed in cellulolytic bacteria (e.g., *Ruminococcus* and *Butyrivibrio* spp.)\(^{(38,39)}\) tended to be less abundant in bacterial cells from AF-fed cows with greater intakes of lignin than the CON cows. No differences, however, were observed in the relative abundances of these genera and no relationship was observed between this FA and bacterial taxa. One explanation for no alterations observed in the bacteria and protozoal cell OBCFA was the lack of change in their community structures. The cellulolytic bacterial genus *Coprococcus* was greater in AF-fed cows, but it was low in abundance in comparison to more abundant genera *Prevotella* and *Butyrivibrio*.

CON- and AF-fed cows had greater relative abundances of the starch-utilizing genus *Entodinium* (48% and 37%, respectively) compared to Jersey cows at 183 DIM.
(23%) as reported by Cersosimo et al. 2016. The starch DMI of AF broadleaf (8.1% DM) was greater than mixed grasses from CON (2.2% DM) and AF (2.7% DM) pastures, but the estimated inclusion of AF broadleaf in the diet was likely not enough to elicit an effect on the abundance of Entodinium and the other protozoal taxa observed in CON and AF-fed cows. Rumen protozoal genera Eremoplastron and Dasytricha were positively correlated with protozoal iso-17:0 suggesting that rumen protozoa may contain different proportions of BCFA than others. The most abundant protozoal genus Entodonium did not correlate with protozoal CLA contents and negatively correlated with VA, whereas Devillard et al.⁴⁰ observed that the species Entodinium caudatum contained CLA while Entodinium furca did not in monofaunated sheep. Cersosimo et al.²³ identified a positive relationship between Metadinium and protozoal CLA contents, but no relationship between Entodinium and CLA or VA contents in three dairy breeds across a lactation period. More research is warranted to investigate what influences the rumen protozoal cell FA compositions and if protozoal species have unique cellular FA compositions.

Although a large proportion of milk OBCFA are derived from ruminal bacteria, the rumen OBCFA profile does not necessarily reflect that identified in milk⁴¹. Vlaeminck et al.⁴² indicated the challenge of using rumen OBCFA as a proxy for milk OBCFA profiles by observing postruminal modifications of the duodenal OBCFA profile. In the present study, the proportion of total rumen bacterial OBCFA content was higher (8.53 g/100g - 9.93 g/100g FA) than the proportion of total milk OBCFA content (3.59 g/100g FA - 3.91 g/100g FA). Factors of which include 1) mode of transportation in blood (e.g., phospholipids or triacylglycerols), 2) absorption rate in the small intestine, 3) de novo synthesis of OCFA in the mammary gland, as well as 4) storage, mobilization,
and metabolism of OBCFA contribute to lower milk OBCFA content and concentrations than the rumen\(^{(41)}\). Partial endogenous carbon-chain elongation results in low transfer efficiencies of \(\text{anteiso}-15:0\) and \(\text{iso}-15:0\) and adds to \(\text{anteiso}-17:0\) and \(\text{iso}-17:0\) in milk contents\(^{(41)}\). Contents of \(\text{anteiso}-17:0\) increased in milk while contents of \(\text{anteiso}-15:0\) decreased in milk from both treatment groups, suggesting carbon-chain elongation, while contents of \(\text{iso}-15:0\) and \(\text{iso}-17:0\) decreased in milk.

Proportions of 17:0 to 15:0 followed the previously outlined 2:1 ratio in milk\(^{(43)}\). As milk contents of 15:0 and 17:0 from AF-fed cows were greater than CON-fed cows, yet no difference in ruminal propionate proportions were observed, endogenous FA synthesis likely contributed. When cows were fed an ensiled buckwheat and Italian ryegrass mixture versus Italian ryegrass, no alterations in 15:0 and 17:0 milk concentrations were observed\(^{(17)}\). Moreover, when reproductive stage buckwheat was fed to Holstein and Brown Swiss cows, the total milk BCFA content was greater than when fed at vegetative stage. Furthermore, the content of total milk BCFA was greater (1.10 g BCFA/kg milk) in Holsteins and Brown Swiss than in Jerseys from the present study (0.75 g BCFA/kg milk).\(^{(44)}\) Greater inclusion of buckwheat in the diet and potentially breed differences were potential contributing factors\(^{(44)}\).

Cows fed AF had higher total SFA and individual milk medium-chain FA (11:0 to 16:0) contents, typically derived from \textit{de novo} FA synthesis in the mammary gland. When whole plant\(^{(44)}\) or ensiled\(^{(17)}\) buckwheat were fed to Holstein and Brown Swiss cows, the total SFA, particularly, contents were lower (24.32 and 31.56 g SFA/kg milk, respectively) than those observed from Jersey cows in the present study (33.66 g SFA/kg milk). Normally, milk from Jersey cows have greater total SFA as well as short and
medium-chain SFA than milk from Holstein cows\textsuperscript{(21,45,46)}, suggesting greater \textit{de novo} FA synthesis activity\textsuperscript{(45)}. Rico \textit{et al.}\textsuperscript{(47)} observed that Holsteins supplemented with PA had lower milk contents (g/100g) of \textit{de novo} FA and greater milk fat yields than those supplemented with SA. Intake of PA did not differ between treatment groups, but milk fat yields were greater in AF-fed cows than CON-fed cows\textsuperscript{(48)}. A survey of US retail milk across different seasons and geographic locations showed similar percentages of PA relative to AF-fed cows (46.7 and 44.1\% of total SFA, respectively)\textsuperscript{(49)}. Recent research demonstrated a linear relationship between \textit{de novo} FA (g/100g FA) and milk fat production\textsuperscript{(50)}. This finding is important to U.S. dairy farmers who are paid premiums for butterfat content and to those interested in using milk for the production of cheese, ice cream, and yogurt.

The average milk 18:2 $c_{9},t_{11}$ content (0.64 g/100g) was similar to the amount observed in a U.S. retail milk survey (0.55 g/100g FA)\textsuperscript{(49)}. No differences in milk 18:2 $c_{9},t_{11}$, ALA, or LA were seen in cows fed whole buckwheat\textsuperscript{(44)}. The Jersey cows from the present study had more 18:2 $c_{9},t_{11}$ per serving of whole milk (41 mg) than Jersey cows fed a TMR (< 30 mg/serving), demonstrating how pasture consumption results in greater milk content of 18:2 $c_{9},t_{11}$\textsuperscript{(21)}. ALA content per serving of whole milk was similar to that of retail milk from England (41 mg/serving)\textsuperscript{(51)}. Recommended intake of n-3 FA for men and women is 1.6 g/d and 1.1 g/d, respectively\textsuperscript{(52)}. However, one serving of whole milk from the CON-fed cows would only contribute to approximately 3\% and 4\% of the daily n-3 requirement for men and women, respectively.

In conclusion, the present study was the first to compare rumen microbial and milk FA profiles from Jersey cows grazing summer AF. Specifically, this study
determined the: 1) FA yield in the diet, 2) microbial FA profiles with emphasis on
ruminal-derived bioactive FA, 3) relative abundances of rumen microbial taxa, and 4)
amount of individual FA per serving of whole milk. Key ruminal VFA, microbial FA and
taxa were not altered. Total milk de novo and mixed FA contents were greater in AF-fed
cows compared to CON cows, while total preformed FA did not differ between treatment
groups. Future steps to consider include 1) increasing the amount of AF offered, 2)
observing regrowth of AF, and 3) measuring the duodenal flow of FA.

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partially financed this study.

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Education SARE grant LNE13-323, and the Graduate Northeast SARE grant GNE15-097
supported the project.

6.8 Conflict of Interest
None.

6.9 Authorship
LC, RT, and KJ collected on-farm data. LC performed PCR, lipid extractions and
analyses, bioinformatics, and wrote the manuscript. AB, LC, and JK contributed to the
conception and design of the study. JK, AB, SG contributed to data interpretation and revised content of the article.

6.10 References


11. Kaneda T (1991) Iso-and anteiso-fatty acids in bacteria: biosynthesis, function,


Table 6-1 Fatty acid compositions of total mixed ration and pasture components offered to lactating Jersey cows

<table>
<thead>
<tr>
<th>Component (g/100g FA)</th>
<th>total FA (%DM&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ALA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ΣSFA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ΣMUFA&lt;sup&gt;e&lt;/sup&gt;</th>
<th>ΣPUFA&lt;sup&gt;f&lt;/sup&gt;</th>
<th>n-6/n-3&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMR</td>
<td></td>
<td>3.19</td>
<td>15.6</td>
<td>2.00</td>
<td>16.4</td>
<td>35.3</td>
<td>26.2</td>
<td>20.0</td>
<td>17.6</td>
<td>62.4</td>
</tr>
<tr>
<td>CON&lt;sup&gt;i&lt;/sup&gt; pasture</td>
<td></td>
<td>2.12</td>
<td>21.3</td>
<td>2.27</td>
<td>3.67</td>
<td>22.3</td>
<td>43.6</td>
<td>28.2</td>
<td>4.75</td>
<td>67.0</td>
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<tr>
<td>Mixed grasses</td>
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<td>2.08</td>
<td>18.4</td>
<td>1.11</td>
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<td>20.0</td>
<td>52.7</td>
<td>23.7</td>
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<td>33.4</td>
<td>4.57</td>
<td>6.12</td>
<td>20.3</td>
<td>25.6</td>
<td>46.1</td>
<td>7.05</td>
<td>46.8</td>
</tr>
<tr>
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<td>1.73</td>
<td>4.11</td>
<td>17.0</td>
<td>50.6</td>
<td>26.0</td>
<td>4.61</td>
<td>69.4</td>
</tr>
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<td>22.8</td>
<td>4.26</td>
<td>6.67</td>
<td>31.6</td>
<td>19.4</td>
<td>38.8</td>
<td>8.14</td>
<td>53.1</td>
</tr>
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<td>2.12</td>
<td>20.2</td>
<td>2.09</td>
<td>4.11</td>
<td>22.0</td>
<td>43.6</td>
<td>27.3</td>
<td>4.94</td>
<td>67.7</td>
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<td>18.6</td>
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<td>2.46</td>
<td>19.5</td>
<td>52.1</td>
<td>24.5</td>
<td>3.04</td>
<td>72.4</td>
</tr>
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<td>Legumes</td>
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<td>28.9</td>
<td>4.39</td>
<td>6.48</td>
<td>21.4</td>
<td>30.2</td>
<td>39.3</td>
<td>7.35</td>
<td>53.3</td>
</tr>
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<td>Weeds</td>
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<td>2.85</td>
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<td>1.37</td>
<td>3.69</td>
<td>18.8</td>
<td>53.5</td>
<td>23.2</td>
<td>3.92</td>
<td>72.9</td>
</tr>
<tr>
<td>Dead material</td>
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<td>22.7</td>
<td>4.01</td>
<td>6.19</td>
<td>29.9</td>
<td>22.5</td>
<td>37.8</td>
<td>7.34</td>
<td>54.9</td>
</tr>
<tr>
<td>Oat</td>
<td></td>
<td>1.79</td>
<td>20.9</td>
<td>1.40</td>
<td>3.41</td>
<td>20.1</td>
<td>48.4</td>
<td>26.4</td>
<td>4.20</td>
<td>69.4</td>
</tr>
<tr>
<td>AF Broadleaf&lt;sup&gt;l&lt;/sup&gt;</td>
<td></td>
<td>1.50</td>
<td>20.5</td>
<td>1.80</td>
<td>6.70</td>
<td>27.3</td>
<td>31.0</td>
<td>27.7</td>
<td>7.87</td>
<td>64.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> DM, dry matter. <sup>b</sup> LA, linoleic acid (18:2<sub>c9,c12</sub>). <sup>c</sup> ALA, α-linolenic acid. <sup>d</sup> ΣSFA, sum of saturated fatty acids (12:0-24:0). <sup>e</sup> ΣMUFA, sum of monounsaturated fatty acids (16:1<sub>c9</sub> + 18:1<sub>c9</sub> + 18:1<sub>c11</sub> + 22:1<sub>c9</sub>). <sup>f</sup> ΣPUFA, sum of polyunsaturated fatty acids (18:2<sub>c9,c12</sub> + 18:3<sub>c9,c12,c15</sub> + 20:2<sub>c11,c14</sub> + 20:3<sub>c5,c8,c11</sub> + 20:3<sub>c11,c14,c17</sub>). <sup>g</sup> n-6 18:2<sub>c9,c12</sub> + 20:2<sub>c11,c14</sub> + 20:3<sub>c5,c8,c11</sub> + 20:3<sub>c11,c14,c17</sub>. <sup>h</sup> Cows fed a TMR, total mixed ration, (60:40 forage: concentrate) and mixed cool-season grasses (n = 8, CON). <sup>i</sup> Cows fed TMR and mixed cool-season grasses plus annual forages buckwheat, chickling vetch, and oat (n = 8, AF). <sup>j</sup> AF broadleaf include buckwheat and chickling vetch.
### Table 6-2 Estimated nutrient and fatty acid intakes of individual dietary components offered to lactating Jersey cows

<table>
<thead>
<tr>
<th></th>
<th>CON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SE</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
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<tr>
<td><strong>Dry matter intake (kg/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19.63</td>
<td>20.41</td>
<td>0.74</td>
<td>ns</td>
</tr>
<tr>
<td>TMR</td>
<td>11.05</td>
<td>11.50</td>
<td>0.34</td>
<td>ns</td>
</tr>
<tr>
<td>Pasture</td>
<td>8.58</td>
<td>8.91</td>
<td>0.79</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Component (kg/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crude protein</td>
<td>2.79</td>
<td>3.06</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>neutral detergent fiber</td>
<td>8.18</td>
<td>8.24</td>
<td>0.38</td>
<td>ns</td>
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<tr>
<td>acid detergent fiber</td>
<td>5.20</td>
<td>5.75</td>
<td>0.26</td>
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<tr>
<td>lignin</td>
<td>0.71</td>
<td>1.17</td>
<td>0.04</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>starch</td>
<td>2.72</td>
<td>2.93</td>
<td>0.08</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Total fatty acids (g FA/d)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>93.5</td>
<td>95.2</td>
<td>3.27</td>
<td>ns</td>
</tr>
<tr>
<td>18:0</td>
<td>11.2</td>
<td>11.3</td>
<td>0.36</td>
<td>ns</td>
</tr>
<tr>
<td>18:1&lt;sub&gt;c9&lt;/sub&gt;</td>
<td>64.5</td>
<td>68.0</td>
<td>1.65</td>
<td>ns</td>
</tr>
<tr>
<td>18:2&lt;sub&gt;c9,c12&lt;/sub&gt; (n-6)</td>
<td>165</td>
<td>171</td>
<td>4.21</td>
<td>ns</td>
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<tr>
<td>18:3&lt;sub&gt;c9,c12,c15&lt;/sub&gt; (n-3)</td>
<td>171</td>
<td>178</td>
<td>6.73</td>
<td>ns</td>
</tr>
<tr>
<td>ΣSFA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>122</td>
<td>125</td>
<td>4.34</td>
<td>ns</td>
</tr>
<tr>
<td>ΣMUFA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>70.9</td>
<td>73.9</td>
<td>1.78</td>
<td>ns</td>
</tr>
<tr>
<td>ΣPUFA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>341</td>
<td>356</td>
<td>11.0</td>
<td>ns</td>
</tr>
<tr>
<td>Σn-6&lt;sup&gt;g&lt;/sup&gt;/Σn-3&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.96</td>
<td>0.96</td>
<td>0.02</td>
<td>ns</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cows fed a TMR, total mixed ration, (60:40 forage: concentrate) and mixed cool-season grasses (n = 8, CON).  
<sup>b</sup> Cows fed TMR and mixed cool-season grasses plus annual forages buckwheat, chickling vetch, and oat (n = 8, AF).  
<sup>c</sup> non-significant (ns), Significance declared at P < 0.05.  
<sup>d</sup> ΣSFA, sum of saturated fatty acids (12:0-24:0).  
<sup>e</sup> ΣMUFA, sum of monounsaturated fatty acids (16:1<sub>c9</sub> + 18:1<sub>c9</sub> + 18:1<sub>c11</sub> + 22:1<sub>c9</sub>).  
<sup>f</sup> ΣPUFA, sum of polyunsaturated fatty acids (18:2<sub>c9,c12</sub> + 18:3<sub>c9,c12,c15</sub> + 20:2<sub>c11,c14</sub> + 20:3<sub>c5,c8,c11</sub> + 20:3<sub>c11,c14,c17</sub>).  
<sup>g</sup> Σn-6, 18:2<sub>c9,c12</sub> + 20:2<sub>c11,c14</sub> + 20:3<sub>c5,c8,c11</sub>,  
<sup>h</sup> Σn-3, 18:3<sub>c9,c12,c15</sub> + 20:3<sub>c11,c14,c17</sub>.  

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Table 6-3 Rumen bacterial and protozoal compositions of selected fatty acids from lactating Jersey cows offered cool-season grass pasture or cool-season grasses plus annual forages

<table>
<thead>
<tr>
<th>Fatty Acid (g/100g FA)</th>
<th>Bacteria</th>
<th>Protozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON(a)</td>
<td>AF(b)</td>
</tr>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-13:0</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td>13:0</td>
<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>iso-14:0</td>
<td>0.70</td>
<td>0.69</td>
</tr>
<tr>
<td>iso-15:0</td>
<td>1.19</td>
<td>1.21</td>
</tr>
<tr>
<td>anteiso-15:0</td>
<td>2.42</td>
<td>2.41</td>
</tr>
<tr>
<td>16:0</td>
<td>2.08</td>
<td>1.95</td>
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<tr>
<td>iso-16:0</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>17:0</td>
<td>17.77</td>
<td>17.32</td>
</tr>
<tr>
<td>iso-17:0</td>
<td>0.41</td>
<td>0.37</td>
</tr>
<tr>
<td>anteiso-17:0</td>
<td>0.18</td>
<td>0.19</td>
</tr>
<tr>
<td>18:0</td>
<td>0.72</td>
<td>0.67</td>
</tr>
<tr>
<td>18:1(t)</td>
<td>47.96</td>
<td>49.27</td>
</tr>
<tr>
<td>18:2(c9),(c12)</td>
<td>72.79</td>
<td>73.99</td>
</tr>
<tr>
<td>18:3(c9),(c12),(c15)</td>
<td>2.99</td>
<td>2.82</td>
</tr>
<tr>
<td>18:1(t)</td>
<td>5.79</td>
<td>5.78</td>
</tr>
<tr>
<td><strong>Unsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1(t)</td>
<td>4.72</td>
<td>4.22</td>
</tr>
<tr>
<td>18:1(c9)</td>
<td>2.40</td>
<td>2.34</td>
</tr>
<tr>
<td>18:2(c9),(c12)</td>
<td>2.07</td>
<td>2.02</td>
</tr>
<tr>
<td>18:3(c9),(c12),(c15)</td>
<td>1.40</td>
<td>1.43</td>
</tr>
<tr>
<td>CLA(c9),(c11)</td>
<td>0.26</td>
<td>0.21</td>
</tr>
<tr>
<td>2(\beta)18:1 trans</td>
<td>5.90</td>
<td>5.32</td>
</tr>
<tr>
<td><strong>SUMFA</strong></td>
<td>12.82</td>
<td>12.18</td>
</tr>
<tr>
<td><strong>ΣCLA</strong></td>
<td>0.38</td>
<td>0.31</td>
</tr>
</tbody>
</table>

\(a\) Cows fed total mixed ration (TMR, 60:40 forage: concentrate) and mixed grasses (n = 8, CON). \(b\) Cows fed TMR and mixed grasses with 5.7% dry matter intake annual forages, buckwheat, chickling vetch, and oat (n = 8, AF). \(c\) Non-significant, NS, significance declared at \(P < 0.05\) and trends at \(0.05 \leq P \leq 0.10\). \(d\) ΣSFA, sum of saturated fatty acids (9:0-24:0). \(e\) ΣOCFA, sum of odd-chain fatty acids (5:0-23:0). \(f\) ΣBCFA, sum of branched-chain fatty acids (iso-13:0 + iso-14:0 + iso-15:0 + anteiso-15:0 + iso-16:0 + iso-17:0 + anteiso-17:0). \(g\) ΣMUFA, sum of monounsaturated fatty acids (16:1-18:1). \(h\) ΣCLA, sum of conjugated linoleic acids.
Table 6-4 Milk content and serving size of selected fatty acids from lactating Jersey cows offered cool-season grass pasture or cool-season grasses plus annual forages

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>CON</th>
<th>AF</th>
<th>SE^a</th>
<th>P-value^b</th>
<th>CON</th>
<th>AF</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4:0</td>
<td>1.18</td>
<td>1.23</td>
<td>0.03</td>
<td>ns</td>
<td>210.2</td>
<td>205.9</td>
<td>4.50</td>
<td>ns</td>
</tr>
<tr>
<td>6:0</td>
<td>0.86</td>
<td>0.92</td>
<td>0.02</td>
<td>0.06</td>
<td>155.4</td>
<td>153.4</td>
<td>2.72</td>
<td>ns</td>
</tr>
<tr>
<td>8:0</td>
<td>0.55</td>
<td>0.58</td>
<td>0.01</td>
<td>0.04</td>
<td>98.7</td>
<td>97.3</td>
<td>1.52</td>
<td>ns</td>
</tr>
<tr>
<td>10:0</td>
<td>1.35</td>
<td>1.45</td>
<td>0.03</td>
<td>0.04</td>
<td>242.8</td>
<td>241.9</td>
<td>3.54</td>
<td>ns</td>
</tr>
<tr>
<td>12:0</td>
<td>1.57</td>
<td>1.74</td>
<td>0.04</td>
<td>0.03</td>
<td>282.1</td>
<td>289.9</td>
<td>6.12</td>
<td>ns</td>
</tr>
<tr>
<td>iso-14:0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.00</td>
<td>ns</td>
<td>8.57</td>
<td>8.22</td>
<td>0.32</td>
<td>ns</td>
</tr>
<tr>
<td>14:0</td>
<td>5.05</td>
<td>5.52</td>
<td>0.10</td>
<td>0.01</td>
<td>909.7</td>
<td>922.7</td>
<td>12.4</td>
<td>ns</td>
</tr>
<tr>
<td>iso-15:0</td>
<td>0.10</td>
<td>0.11</td>
<td>0.00</td>
<td>ns</td>
<td>18.6</td>
<td>18.0</td>
<td>0.44</td>
<td>ns</td>
</tr>
<tr>
<td>15:0</td>
<td>0.41</td>
<td>0.44</td>
<td>0.01</td>
<td>0.08</td>
<td>74.1</td>
<td>73.2</td>
<td>1.60</td>
<td>ns</td>
</tr>
<tr>
<td>iso-16:0</td>
<td>0.12</td>
<td>0.12</td>
<td>0.00</td>
<td>ns</td>
<td>21.6</td>
<td>20.4</td>
<td>0.80</td>
<td>ns</td>
</tr>
<tr>
<td>16:0</td>
<td>12.70</td>
<td>15.73</td>
<td>0.59</td>
<td>0.02</td>
<td>2378</td>
<td>2528</td>
<td>32.8</td>
<td>0.02</td>
</tr>
<tr>
<td>iso-17:0</td>
<td>0.13</td>
<td>0.14</td>
<td>0.00</td>
<td>ns</td>
<td>24.5</td>
<td>22.6</td>
<td>0.78</td>
<td>ns</td>
</tr>
<tr>
<td>anteiso-17:0</td>
<td>0.10</td>
<td>0.10</td>
<td>0.00</td>
<td>ns</td>
<td>18.7</td>
<td>17.0</td>
<td>0.78</td>
<td>ns</td>
</tr>
<tr>
<td>17:0</td>
<td>0.24</td>
<td>0.26</td>
<td>0.00</td>
<td>0.07</td>
<td>43.6</td>
<td>42.6</td>
<td>0.76</td>
<td>ns</td>
</tr>
<tr>
<td>18:0</td>
<td>5.19</td>
<td>5.06</td>
<td>0.27</td>
<td>ns</td>
<td>944.5</td>
<td>836.1</td>
<td>37.0</td>
<td>0.05</td>
</tr>
<tr>
<td>ΣSFA^c</td>
<td>30.01</td>
<td>32.75</td>
<td>0.63</td>
<td>0.03</td>
<td>5429</td>
<td>5427</td>
<td>30.5</td>
<td>ns</td>
</tr>
<tr>
<td>ΣOCFA^d</td>
<td>0.89</td>
<td>0.96</td>
<td>0.02</td>
<td>0.03</td>
<td>161.0</td>
<td>160.3</td>
<td>2.16</td>
<td>ns</td>
</tr>
<tr>
<td>ΣBCFA^e</td>
<td>0.72</td>
<td>0.74</td>
<td>0.02</td>
<td>ns</td>
<td>130.4</td>
<td>123.4</td>
<td>3.07</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Unsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 t11</td>
<td>0.79</td>
<td>0.78</td>
<td>0.04</td>
<td>ns</td>
<td>143.5</td>
<td>129.6</td>
<td>7.26</td>
<td>ns</td>
</tr>
<tr>
<td>18:2 c9,c12 (n-6)</td>
<td>6.33</td>
<td>6.48</td>
<td>10.20</td>
<td>ns</td>
<td>1173</td>
<td>1153</td>
<td>15.3</td>
<td>ns</td>
</tr>
<tr>
<td>18:3 c9,c12,c15 (n-3)</td>
<td>0.52</td>
<td>0.54</td>
<td>0.02</td>
<td>ns</td>
<td>94.4</td>
<td>89.2</td>
<td>3.12</td>
<td>0.09</td>
</tr>
<tr>
<td>CLA c9,t11</td>
<td>0.23</td>
<td>0.24</td>
<td>0.01</td>
<td>ns</td>
<td>41.4</td>
<td>41.3</td>
<td>1.31</td>
<td>ns</td>
</tr>
<tr>
<td>ΣMUFA^f</td>
<td>8.78</td>
<td>9.17</td>
<td>0.23</td>
<td>ns</td>
<td>1563</td>
<td>1565</td>
<td>2.15</td>
<td>ns</td>
</tr>
<tr>
<td>ΣPUFA^g</td>
<td>1.35</td>
<td>1.44</td>
<td>0.06</td>
<td>ns</td>
<td>243.3</td>
<td>243.8</td>
<td>10.4</td>
<td>ns</td>
</tr>
<tr>
<td>ΣCLA^h</td>
<td>0.30</td>
<td>0.32</td>
<td>0.02</td>
<td>ns</td>
<td>54.3</td>
<td>54.2</td>
<td>3.76</td>
<td>ns</td>
</tr>
<tr>
<td>Σn-6^i</td>
<td>0.58</td>
<td>0.62</td>
<td>0.02</td>
<td>ns</td>
<td>106.6</td>
<td>102.0</td>
<td>4.11</td>
<td>ns</td>
</tr>
<tr>
<td>Σn-3^k</td>
<td>0.28</td>
<td>0.30</td>
<td>0.01</td>
<td>ns</td>
<td>50.3</td>
<td>50.6</td>
<td>1.97</td>
<td>ns</td>
</tr>
</tbody>
</table>
Cows fed TMR, total mixed ration (60:40 forage: concentrate) and mixed grasses (n = 8, CON), \(^a\) Cows fed TMR and mixed grasses plus annual forage crops (n = 8, AF), \(^b\) ns, non-significant, significance declared at \(P < 0.05\), trends at \(0.5 \leq P \leq 0.10\), \(^c\) \(\Sigma\) SFA, sum of saturated fatty acids (4:0-24:0); \(^d\) \(\Sigma\) OCFA, sum of odd-chain fatty acids (5:0-23:0); \(^e\) \(\Sigma\) BCFA, sum of branched-chain fatty acids (iso-13:0 + anteiso-13:0 + iso-14:0 + iso-15:0 + anteiso-15:0 + iso-16:0 + iso-17:0 + anteiso-17:0 + iso-18:0); \(^f\) \(\Sigma\) MUFA, sum of monounsaturated fatty acids (14:1-22:1); \(^g\) \(\Sigma\) PUFA, sum of polyunsaturated fatty acids (18:2-22:5); \(^h\) \(\Sigma\) CLA, sum of conjugated linoleic acids; \(^i\) \(\Sigma\) n-6, 18:3 c9, c12, c15 + 20:5 c5, c8, c11, c14, c17 + 22:5 c7, c10, c13, c16, c19; \(^j\) \(\Sigma\) n-3, 18:2 c9, c12 + 18:3 c6, c9, c12 + 20:2 c11, c14 + 20:3 c5, c8, c11 + 20:4 c5, c8, c11, c14.
Figure 6-1 Distribution of rumen bacterial 16S and protozoal 18S rRNA genes to nearest valid taxa from lactating Jersey cows offered cool-season grass pasture or cool-season grasses plus annual forages

(A) Relative abundance of protozoal genera by targeting the V3-V4 region of the 18S rRNA gene, (B) Relative abundance of bacterial genera belonging to the phylum Firmicutes by targeting the V1-V3 region of the 16S rRNA gene, (C) Relative abundance of bacterial genera belonging to the phylum Bacteroidetes by targeting the V1-V3 region of the 16S rRNA gene, CON, control, Cows fed TMR, total mixed ration (60:40 forage: concentrate) and mixed grasses (n = 8), AF, annual forage, Cows fed TMR and mixed grasses plus annual forages buckwheat, chickling vetch, and oat (n = 8), Significance declared at *P < 0.05, Trends at † 0.5 < P < 0.10, g, genus, f, family, o, order.
Figure 6-2 Corrplot demonstrating the relationship between rumen protozoal genera and cellular fatty acids from lactating Jersey cows offered traditional pasture or annual forage crops plus traditional pasture.

The corrplot depicts Pearson’s correlations between rumen protozoal genera and cellular fatty acids, FA. Significant correlations (P < 0.05) between protozoal genera and FA are depicted with black squares. The legend values -1 to 1 are the Pearson’s correlation coefficients. SFA, saturated FA, MUFA, monounsaturated FA, PUFA, polyunsaturated FA, CLA, conjugated linoleic FA, OCFA, odd-chain FA, BCFA, branched-chain FA.
Supplementary Table 6-5 Proportions of rumen volatile fatty acids from lactating Jersey cows consuming cool-season grass/legume pasture or annual forages plus cool-season grass/legume pasture

<table>
<thead>
<tr>
<th>volatile fatty acid (%)</th>
<th>CON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SE</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td>71.2</td>
<td>71.3</td>
<td>0.38</td>
<td>ns</td>
</tr>
<tr>
<td>propionate</td>
<td>15.7</td>
<td>15.9</td>
<td>0.32</td>
<td>ns</td>
</tr>
<tr>
<td>butyrate</td>
<td>11.0</td>
<td>10.6</td>
<td>0.21</td>
<td>ns</td>
</tr>
<tr>
<td>isobutyrate</td>
<td>0.98</td>
<td>0.82</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>valerate</td>
<td>0.66</td>
<td>0.73</td>
<td>0.08</td>
<td>ns</td>
</tr>
<tr>
<td>isovalerate</td>
<td>0.56</td>
<td>0.61</td>
<td>0.12</td>
<td>ns</td>
</tr>
<tr>
<td>acetate:propionate</td>
<td>4.59</td>
<td>4.47</td>
<td>0.12</td>
<td>ns</td>
</tr>
<tr>
<td>Total VFA&lt;sup&gt;d&lt;/sup&gt; (mM)</td>
<td>72.5</td>
<td>80.9</td>
<td>5.14</td>
<td>ns</td>
</tr>
</tbody>
</table>

<sup>a</sup> control, CON (n = 8) cows consuming total mixed ration plus cool-season grass/legume pasture,<sup>b</sup> annual forage, AF cows (n = 8) fed total mixed ration plus cool-season grass/legume pasture, buckwheat, chickling vetch, and oats,<sup>c</sup> ns, non-significant, significance declared at P < 0.05,<sup>d</sup> VFA, volatile fatty acids
Supplementary Table 6-6 Rumen bacterial and protozoal diversity and density from lactating Jersey cows consuming cool-season grass/legume pasture or cool-season grass/legume plus annual forages

<table>
<thead>
<tr>
<th>Diversity Measure</th>
<th>Bacteria</th>
<th>SE</th>
<th>P-value</th>
<th>Protozoa</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON(^b)</td>
<td>AF(^c)</td>
<td></td>
<td>CON</td>
<td>AF</td>
<td></td>
</tr>
<tr>
<td>OTU(^d)</td>
<td>642</td>
<td>652</td>
<td>15.6</td>
<td>4.93</td>
<td>3.39</td>
<td>0.99</td>
</tr>
<tr>
<td>Good’s Coverage (%)</td>
<td>80.9</td>
<td>80.2</td>
<td>0.01</td>
<td>99.9</td>
<td>99.9</td>
<td>3.79 x 10(^-4)</td>
</tr>
<tr>
<td>Shannon Diversity Index</td>
<td>3.97</td>
<td>4.05</td>
<td>0.19</td>
<td>0.02</td>
<td>0.02</td>
<td>3.60 x 10(^-3)</td>
</tr>
<tr>
<td>Inverse Simpson Index</td>
<td>6.68</td>
<td>8.37</td>
<td>1.29</td>
<td>1.00</td>
<td>1.00</td>
<td>9.82 x 10(^-4)</td>
</tr>
<tr>
<td>Density(^e)</td>
<td>9.23</td>
<td>9.02</td>
<td>0.27</td>
<td>3.77</td>
<td>3.47</td>
<td>0.19</td>
</tr>
</tbody>
</table>

\(^a\)ns, non-significant, significance declared at \(P < 0.05\), \(^b\) control, CON (n = 8) cows consuming total mixed ration plus cool-season grass/legume pasture, \(^c\) annual forages, AF cows (n = 8) fed total mixed ration plus cool-season grass/legume pasture, buckwheat, chickling vetch, and oats, \(^d\) OTU, operational taxonomic units, \(^e\) bacterial density as \(\log_{10}\) bacterial 16S rRNA copies/mL whole rumen digesta, protozoal density as \(\log_{10}\) cells/mL whole rumen digesta
Chapter 7. Conclusions

My dissertation work accomplished the initial goal of using NGS techniques to determine if breed and lactation stage affect rumen methanogen and protozoal communities, as well as protozoal cell FA in dairy cows. Once the objectives of Chapters 2 and 3 were achieved, a more applicable on-farm approach was applied to determine if the supplementation of cool-season grass pastures with AF during typical periods of decreased pasture mass alters the rumen environment, production performance, or the milk FA profile provided to the consumer. In addition to accomplishing my dissertation research goals, my work i) filled several gaps in knowledge regarding less abundant rumen microbiota, ii) had significant implications for dairy farmers, rumen microbiologists and nutritionists, and dairy consumers, and iii) identified research limitations (e.g., DMI estimations) that indicate a need to pursue further research.

7.1 Implications

Although results from Chapters 2 and 3 demonstrated that the rumen methanogen and protozoal diversity measures differed at 3 DIM (transition period) than from those at 93, 183, and 273 DIM, and less so by breed, this study did not offer practical on-farm applications to be used by dairy farmers. Instead, novel information was provided about the less abundant rumen microbiota from three breeds of dairy cows during the transition period and across a lactation period. This insight, along with our current knowledge about rumen bacteria, can be used towards the prevention of metabolic disorders (i.e., subacute ruminal acidosis) that typically contribute to reduced farm revenue. To the author’s best knowledge, this study was the first to report the
persistence of a core rumen methanogen community, and to establish that dairy breed and lactation stage are important factors contributing to the protozoal cell FA profile. By identifying a core methanogen community of four methanogen species, focus can be directed on these methanogens to determine why these four species form a community and how or if they co-occur and interact with rumen bacteria, protozoa, and/or fungi. Furthermore, this observational study used current NGS and GLC techniques informing the scientific community about the less-studied rumen protozoa and their cell FA compositions from lactating dairy cows. This study was a starting point for future research to expand upon, with the overall goals of 1) gaining more knowledge about rumen ecology and function and 2) potentially enhancing the bioactive FA profile of milk fat.

Chapters 4-6 not only focused on the rumen microbial communities, but also on applied alternative on-farm feeding strategies of particular interest to dairy farmers in the Northeastern US. These experiments demonstrated that short-term grazing did not alter the abundant rumen protozoal taxa along with their FA and ruminal VFA, suggesting maintenance of a normal rumen environment and health. Contents of total de novo and mixed FA per serving of whole milk were greater in AF-fed cows while preformed FA did not differ, indicating changes as a result of fat mobilization and mammary gland FA synthesis. Notably, milk fat % was greater in AF-fed cows than CON-fed cows in the summer experiment. Because farmers are paid additional milk quality premiums for butterfat, the farmer could receive a greater amount of revenue reflected in the milk check from AF-fed cows. Additionally, an increase in milk fat % could be of interest to ice cream and cheese producers interested in improving the texture and flavor of their
products (Rios et al., 2014, Felfoul et al., 2015). Furthermore, the observed positive relationship between the protozoal genus *Diplodinium* and milk fat% indicates the need to better understand the role protozoa potentially play in modulating milk fat synthesis and animal performance. This study, however, used Jersey cows, which indicates the need for research to determine if the milk fat % would also increase in Holstein cows fed summer AF.

### 7.2 Limitations and Future Approaches
While my dissertation research objectives were reached, there were several limitations that need to be addressed and improved upon by future research approaches. First, amplicon-based sequencing technologies demonstrate several limitations from PCR primer bias to providing sufficient resolution for describing richness and taxa (Li et al., 2016a). A Nature Reviews article showed that full-length archaeal and bacterial 16S rRNA gene sequences (~1540 bp, nine hypervariable regions) are necessary for the most accurate estimations of species richness and classification to genus and species levels (Yarza et al., 2014). As a result of cost and technology restraints, partial 16S rRNA gene sequences are alternatively used. Universally acceptable 16S rRNA hypervariable regions and similarity thresholds have not yet been identified (Janda and Abbott, 2007). Four years after this initial report, the archaeal and bacteria V1-V3 hypervariable region (~467 bp) was, however, recommended as a target region in comparison to other variable regions and full-length sequences (Kim et al., 2011a) and was suggested to offer a more detailed assessment of diversity and community ecology than the V3-V4 region (Zheng et al., 2015). Keeping in mind these limitations, PCR (e.g., primers, number of cycles) and bioinformatics workflows (e.g., similarity thresholds) were maintained across all of my
experiments and archaeal and bacterial primers targeted the V1-V3 hypervariable region of the 16S rRNA gene.

With respect to the protozoal 18S rRNA gene, it was suggested by Newbold et al. (2015) that NGS technologies may not be the most accurate when reporting alpha diversity (i.e., within sample) such as rarefaction curves or relative abundances of taxa, but are reliable when reporting beta diversity (i.e., between samples), such as PCoA analyses. This is because larger (by volume) protozoa like, *Epidinium caudatum* have five times more 18S rRNA gene copies than *Entodinium caudatum* (Sylvester et al., 2009). It was suggested by Newbold et al. that microscopy is the gold standard for protozoal genus and species identifications, however, this technique is laborious and requires a high level of experience not accomplished by this dissertation work.

Measuring DMI was one of the major challenges of my research experiments. In Chapters 2-3, individual DMI measurements were not achieved, as the cows were co-housed in free stalls. In Chapters 4-6 individual DMI of TMR was measured with the use of the Calan door system, but individual pasture intakes of AF were measured by using chromium oxide. With respect to Chapters 2-3, chromium oxide could have been one potential solution to estimate individual DMI of TMR and production efficiency (ECM/DMI) by breed and over a lactation period. Furthermore, the individual DMI of starch within the TMR could have potentially brought insight into why Holsteins had greater relative abundances of the starch-utilizing protozoal genus *Entodinium* than Jerseys and Holstein-Jersey crossbreeds. Although previous studies showed differences in rumen bacteria and DMI between Holsteins and Jerseys (Paz et al., 2016), it is important for future work to better define the relationship between DMI and production efficiency.
with rumen methanogen and protozoal communities. With respect to Chapters 4-6, using the “cut and carry method” or using only one type of AF, instead of a mixture of three or five species, would have made pasture DMI more accurate and potentially less subject to individual animal selectivity.

Throughout all of my research experiments, whole rumen digesta samples were collected by esophageal intubation, rather than by rumen cannula. Several advantages to esophageal intubation over using rumen cannulas include increased animal numbers and statistical power, and decreased cost and invasiveness. Concerns about esophageal intubation relate to saliva contamination, inconsistent position of the tube in the rumen, and adequate recovery of both solid and liquid rumen material. However, several studies indicated that esophageal intubation is a sufficient method for estimating rumen bacterial populations (Lodge-Ivey et al., 2009; Paz et al., 2016), which does not necessarily indicate that it is an adequate method for describing rumen methanogen and protozoal communities or microbial FA profiles. Notably, Tapio et al. 2016 demonstrated that rumen bolus or buccal samples are non-invasive predictors of the rumen microbial community (i.e., bacteria, archaea, protozoa, and fungi) across five treatments. This novel technique allows researchers to sample more animals and describes a microbial phenotype. If rumen microbial diversity analyses become more cost-effective and less time consuming than they currently are, this technique may be of interest to dairy farmers for future on-farm application. For example, if we establish which rumen microbiota are associated with feed efficiency or indicators of rumen-associated metabolic disorders (e.g., *Megasphaera elsdenii* with ruminal acidosis), farmers or veterinarians could collect a bolus sample to target specific microbial species and determine if the rumen is
functioning normally. It would also be worthwhile to observe if the bolus sample could be used to estimate the microbial FA profile or to measure certain rumen-derived FA (e.g., 18:2 t10, c12) associated with milk fat depression (Rico et al., 2015).

My dissertation research identified what rumen protozoa and methanogen taxa are present in dairy cows and the factors that may alter their relative abundances, but it is also important to understand why specific microbiota exist in the rumen. Because culture-dependent strategies to isolate rumen microbiota are challenging, future approaches could incorporate shotgun metagenomic techniques as described by Pitta et al. 2016. These techniques would not only characterize the rumen microbiome, but also define the metabolic functionality of the less abundant rumen microbiota in relation to animal performance and provide insight as to why these microbiota are present.
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Appendix A-1

The following results are in addition to those presented in Chapter 2: Breed And Lactation Stage Alter The Rumen Protozoal Fatty Acid Profiles And Community Structures In Primiparous Dairy Cattle.

Correlation Analyses

The CORR procedure in SAS (v. 9.4) was used to calculate Pearson’s correlation coefficients between rumen protozoal genera and production performance by breed (Holstein, Jersey, and Holstein-Jersey Crosses). The corrplot package in R Studio was used to visualize the correlations. Milk component data was reported by Bainbridge et al. 2016a.

Results

Significant breed by DIM interactions were not observed for protozoal genera and milk components (Chapter 2 and Bainbridge et al, 2016a). Milk fat and protein percentages, however, were not included because significant breed by DIM interactions were observed (Bainbridge et al, 2016a).

Protozoa belonging to the genus Entodinium from Holstein cows correlated \((P < 0.01)\) to milk \((r = 0.57)\), milk fat \((r = 0.49)\) and milk protein \((r = 0.49)\) yields, ECM \((r = 0.53)\), and FCM \((r = 0.53)\), whereas protozoa belonging to the genus Eudiplodinium were negatively correlated to protein yield \((r = 0.53, P < 0.01)\).

Protozoa belonging to the less abundant genus Isotricha (1-4% relative abundance) from Jersey cows correlated to milk fat yield \((r = 0.39, P = 0.02)\), FCM \((r = 0.37, P = 0.04)\), and ECM \((r = 0.36, P = 0.04)\), while no other correlations were identified in Jersey cows.
Protozoa belonging to *Epidinium* from Holstein-Jersey Crossbred cows correlated with milk fat yield ($r = 0.46$, $P = 0.01$), while protozoa belonging to unclassified members of the family Orphryoscolecidae negatively correlated with milk yield ($r = -0.41$, $P = 0.03$).

**Discussion**

In addition to determining if dairy breed affects rumen protozoal community structures across a lactation period, this experiment is one of the first to observe relationships between rumen protozoal genera and production performance. Shimado et al. [1988], to the author’s knowledge, is the only study to perform correlation analyses between rumen protozoa and animal performance. They observed a positive correlation between the protozoal genus *Dasytricha* and milk fat % and suggested that this genus may be important to recovery from milk fat depression, however did not discuss the relationship further. In the present study, the abundance of *Dasytricha* was low (< 1%) and did not correlate with production measurements from any breed.

The relationship between the starch-utilizing protozoal genus *Entodinium* and milk, milk fat and protein yields in Holstein may have been a result of increased starch DMI in comparison to Jersey and Holstein-Jersey Crossbred cows. The correlations observed between *Isotricha* and milk production and components may have been influenced by the ability of *Isotricha* spp. to convert the substrates, fructan, fructose, glucose, and sucrose to glycogen. It was suggested by Hall et al. [2011] that the proportion of these substrates converted to glycogen may decrease the production rates of microbial cells and proteins (contributors to milk fat and protein). Currently, the correlations presented here are speculative and indicate that further research is needed to
determine how rumen protozoa contribute (if at all) to animal performance and if host genetics influences this relationship.
Figure A1-1 Corrplot demonstrating the relationship between rumen protozoal genera and production performance from primiparous Holstein cows.

The corrplot depicts Pearson’s correlations between rumen protozoal genera (% relative abundance) and production performance from Holstein cows (n = 7) at 3, 93, 183, and 273 DIM. Significant correlations (P < 0.05) between protozoal genera and production performance are depicted with black square outlines. The legend values -1 to 1 are the Pearson’s correlation coefficients.
Figure A1-2 Corrplot demonstrating the relationship between rumen protozoal genera and production performance from primiparous Jersey cows.

The corrplot depicts Pearson’s correlations between rumen protozoal genera (% relative abundance) and production performance from Jersey cows (n = 8) at 3, 93, 183, and 273 DIM. Significant correlations (P < 0.05) between protozoal genera and production performance are depicted with black square outlines. The legend values -1 to 1 are the Pearson’s correlation coefficients.
Figure A1-3 Corrplot demonstrating the relationship between rumen protozoal genera and production performance from primiparous Holstein-Jersey Crossbred cows.

The corrplot depicts Pearson’s correlations between rumen protozoal genera (% relative abundance) and production performance from Holstein-Jersey Crossbred cows (n = 7) at 3, 93, 183, and 273 DIM. Significant correlations (P < 0.05) between protozoal genera and production performance are depicted with black square outlines. The legend values -1 to 1 are the Pearson’s correlation coefficients.