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ANALYSIS OF THE EFFECTS OF FEEDING REGIMENS THAT FEED TWO RATIONS
OVER THE DAY ON RUMEN FERMENTATION PRODUCTS AND RUMEN MICROBIAL
POPULATIONS IN DAIRY CATTLE

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ABSTRACT

There is a daily pattern of feed intake that results in a change in rumen digesta composition over the day. The object of this study was to investigate the fatty acid composition of rumen digesta and the relative abundance of selected microbial populations in the rumen over the day when cows are fed the same diet for the entire day or diets differing in forage concentration. Samples were analyzed from a previous experiment using nine ruminally cannulated multiparous Holstein cows (158 + 48 DIM, mean + SD) in a 3x3 Latin Square design. Three diets used included a control (CON; 33.3% NDF), a low fiber diet (LF; 29.6% NDF), and a high fiber diet (HF; 34.8% NDF). The control treatment (CON) was fed the control diet at 0900 h, the high/low treatment (H/L) was fed HF at 0900 h and LF at 2200 h, and the low/high (L/H) treatment was fed LF at 0900 h and HF at 1300 h. Rumen samples were collected 8 times during the end of each period to represent every 3 h over the day. Whole rumen digesta was freeze dried and methylated prior to fatty acid analysis by gas chromatography. Additionally, microbial DNA was extracted from 6 of the cows and 10 microbial populations analyzed by Real-Time PCR. Preplanned contrasts compared CON to H/L and H/L to L/H at each time point over the day. There was a time interaction for all fatty acids except *cis*-10 *trans*-12 CLA and a treatment by time interaction for C18:0 and *cis*-9 C18:1. There was an effect of time, but no treatment by time interaction for most microbial populations other than S*treptococcus bovis*. *Megasphaera elsdenii*, *B. fibrisolvens/Pseudobutyrivibrio*, and total anaerobic fungi saw no effect. There is a daily rhythm of ruminal fatty acids that is impacted by diet composition, but the daily rhythm of microbial populations is less impacted.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

B. fibrisolvens	*B. fibrisolvens/Pseudobutyrivibrio*
B. hungatei	*Butytivibrio hungatei*
CLA	Conjugated Linoleic Acid
CON	Control diet
CP	Crude Protein
DIM	Days In Milk
DM	Dry Matter
DMI	Dry Matter Intake
F. succinogenes	*Fibrobacter succinogenes*
FA	Fatty Acid
FFA	Free Fatty Acid
HF	High Fiber diet
H/L	High/Low treatment
LF	Low Fiber diet
L/H	Low/High treatment
M. elsdenii	*Megasphaera elsdenii*
NDF	Neutral Detergent Fiber
P. bryantii	*Prevotella bryantii*
R. albus	*Ruminococcus albus*
PUFA	Poly-unsaturated Fatty Acid
S. bovis	*Streptococcus bovis*
S. ruminantium	*Selenomonas ruminantium* group
TMR	Total Mixed Ration
VFA	Volatile Fatty Acid

Chapter 1

INTRODUCTION

Cattle have naturally occurring patterns of feed intake, with higher rates of intake occurring around dawn and dusk. These varying rates of intake cause fluctuations in the rate of nutrients entering the rumen and change the composition of rumen digesta. Researchers have realized that different diets result in alterations in ruminal environment and fermentation pattern, most notably a decrease in pH and a decrease in the acetate to propionate molar ratio. *Trans-11* C18:1 and *cis-9, trans-11* CLA are the predominant *trans-* fatty acid (FA) intermediates produced from the ruminal metabolism of linoleic acid under normal conditions; however, ruminal biohydrogenation pathways are dynamic, allowing the production of a wide range of positional and geometric isomers, as well as modified FAs such as hydroxy and keto derivatives.

The natural pattern of feed intake creates a large variation in the amount of fermentable carbohydrate entering the rumen over the day. Daily fluctuation in FA production indicates that the rumen undergoes patterns of fermentation over the day. This has been supposed by a number of other articles. The predominant metabolic pathways and the microbial capacity for isomerization and biohydrogenation depend on the microbial population and the ruminal environment. Dietary factors that affect ruminal fermentation (e.g. high carbohydrate fermentability, high oil, rumensin) modify ruminal FA metabolism through complex associative effects that result in altered ruminal microbial populations, altered pathways of polyunsaturated

fatty acid (PUFA) biohydrogenation, and ruminal outflow of a wide range of biohydrogenation intermediates.

Traditionally, rumen microbes have been classified into groups based on their preferred substrate or product. Although there are thousands of specific species of bacteria in the rumen, changes in members of each niche is a simple approach providing insight into adaptation of microbes with different metabolic requirements and advantages. A previous study by Mullins et al. (2013), collected rumen samples four hours after feeding (PM; 1530 h, high rumen fill) and 2 hours before feeding (AM; 0930 h, low rumen fill). The relative quantity of *Fibrobacter succinogenes* (P=0.03), *Ruminococcus albus* (P=0.05), and total anaerobic fungi (P=0.01) were different between time points. This data supports time of day as a factor important to relative changes in the microbial populations, which ultimately may be the reason for changes in the amount and specific isomers of biohydrogenation products that flow out of the rumen. It is now necessary to look at a greater number of time points throughout the day and investigate the effect of diet composition to more fully understand the daily changes in ruminal microbial populations.

This study investigates the changes over the day of microbial populations selected to represent specific niches in the rumen and determine their association with ruminal fatty acid profile across the day. The goal of this research is to determine the optimal diet concentrations of fiber and starch for each phase of the day to optimize the ruminal environment and microbial fermentation. Modifying ruminal fatty acid metabolism is of great importance because bioactive *trans-* intermediates of ruminal fermentation have a large impact on milk fat synthesis. Therefore, the object of this study was to investigate the fatty acid composition of rumen digesta

and the relative abundance of selected microbial populations in the rumen over the day when cows are fed the same diet for the entire day or diets differing in forage concentration in the morning and evening.

Chapter 2

LITERATURE REVIEW

Rumen Microbial Populations

There is an increasing interest in microbial communities resident in the gastrointestinal tracts of many vertebrate animals due to their critical roles in the nutrition and health of their hosts. Specifically in ruminants, extensive digestion of feed takes place initially in the rumen by multiple microbial species. Here, microbes play a key role in the breakdown of feed components such as starch and fiber in order to provide energy for the host for maintenance and production and allows the utilization of fibrous feeds.

The bovine rumen undergoes substantial changes in environmental conditions and substrate available during the animal's feeding cycle. It is estimated that there are about one thousand billion (10^{12}) microbes per milliliter of digesta found within the stomach of a ruminant. A longstanding challenge in the field of rumen microbiology has been the fact that the classical culturable bacterial species represent only a small fraction of this total rumen microbiome. However, specific well-characterized bacterial species tend to represent the niche they occupy and demonstrate the temporal adaptation in the rumen (Rico et al., 2013). Welkie et al. (2010) observed that cows having substantially different ruminal bacterial community compositions could have similar ruminal microbial outputs, confirming there is considerable niche overlap and capacity for niche replacement. Niches are based on their predominant substrate or enzymatic

activity including amyloytic bacteria, lactate using bacteria, cellulolytic bacteria, and bacteria involved in ruminal fatty acid (FA) biohydrogenation. Other microorganisms found within the rumen microbiome include anaerobic fungi and ciliate protozoa. Importantly, this is a simplification as some species utilize multiple substrates, but simplification is important to providing insight into major changes that occur with treatments.

Amyloytic bacteria (*Streptococcus bovis* and *Prevotella bryantii*) make up a significant part of the rumen's bacterial population, especially when large amounts of starches and sugars are fed in the diet. These nutrients are used by the bacteria as a fuel source and in return produce lactic acid. However, lactate is seldom detected as a major fermentation product of mixed anaerobic communities, such as the rumen, due to a wide variety of lactate-using bacteria (*Megasphaera elsdenii* and *Selenomonas ruminantium* group). Lactate-using bacteria utilize lactic acid to grow and convert it largely to acetate and propionate.

Anaerobic rumen cellulolytic bacteria, protozoa and fungi mainly degrade fibrous material, allowing ruminants to utilize plant fiber for nutrition. Bacteria (*Ruminococcus albus* and *Fibrobacter succinogenes*) are the most abundant form of fiber-digesting microorganisms. Anaerobic rumen fungi produce enzymes that hydrolyze cellulose and xylans, effectively splitting fibrous material apart and making it easier for bacteria to access it (Castillo-Gonzáleza et al., 2014). Higher numbers of fungi are usually found in the rumen of cows fed poorly digestible forages. Ciliate protozoa are mainly found in the slower moving fibrous mat of the rumen, working to digest components of the feed found there (Castillo-Gonzáleza et al., 2014).

Finally, many species of bacteria are involved in rumen fatty acid biohydrogenation and *Butyrivibrio/ Pseudobutyrivibrio* group and *Butytivibrio hungatei* group are well characterized species important to this process. Two microbial transformations seen in the rumen include lipolysis, which causes the release of free fatty acids (FFA) from esterified plant lipids, followed by biohydrogenation, which reduces the number of double bonds (Jenkins, 1993). Biohydrogenation is a multiple step process with *cis-* double bonds first isomerized to *trans-* bonds followed by removal of one double bond at a time. Through this process, lipids are modified by microbial fermentation from dietary unsaturated FA to saturated FA in the rumen.

Fatty Acids in the Diet

The concentration and fatty acid profile of a feed can vary greatly dependent on the ingredients that are included in that feed. In general, oilseeds are usually found to be high in fat content, made up mostly of triglycerides. These concentrates are commonly higher in C18:1 and C18:2 FA, other than flaxseed oil. Opposite of this, forages are usually low in fat content and are made up mostly of complex lipids. Forages are largely associated with higher C18:3 FA content.

Importance of Fatty Acids to the Cow

Fatty acids are a primary component of lipids and are important in a number of biological functions, such as the formation of cell membranes in the form of phospholipids to maintain the fluidity and functionality of the membrane. These compounds are important energy substrates within the body and can be stored in excess amounts in adipose tissue. Fatty acids are composed of a carboxylic acid attached to either a saturated or unsaturated carbon chain, which varies in

length. Most fatty acids made by plants and animals have an even number of carbon atoms, as they are synthesized in two-carbon units. Microbes synthesize some odd and branch chain fatty acids and odd chain fatty acids in small amounts in ruminants by initiation of FA synthesis with an odd number of carbons.

Microbial Fatty Acid Production

Recent advances in ruminal lipid metabolism have focused primarily on the manipulation of physiochemical events in the bovine rumen, aimed at regulating microbial biohydrogenation pathways to alter the absorption of selected FA that might enhance performance or reduce saturation of meat and milk products (Jenkins, 1993). As noted previously, the two most important microbial transformations seen in the rumen are lipolysis and biohydrogenation of FA. Shortly after esterified plant lipids are consumed, they are hydrolyzed extensively by microbial lipases releasing FFA (Jenkins, 1993). Unsaturated FFA are then rapidly hydrogenated by microbes to produce more saturated end products. The initial step in biohydrogenation is an isomerization reaction that converts the *cis*-12 double bond in unsaturated FA to a *trans-11* isomer. Figure 1 provides further detail as an example to the complete hydrogenation of linoleic acid to C18:0.

Esterified Plant Lipid

| Lipases
| Galactosidases
| Phospholipases
↓

Unsaturated Free FA
(e.g. *cis-9, cis-12,* C18:2)

| Isomerase
↓

cis-9, trans-11 C18:2

| Reductase
↓

trans-11 C18:1

| Reductase
↓

C18:0
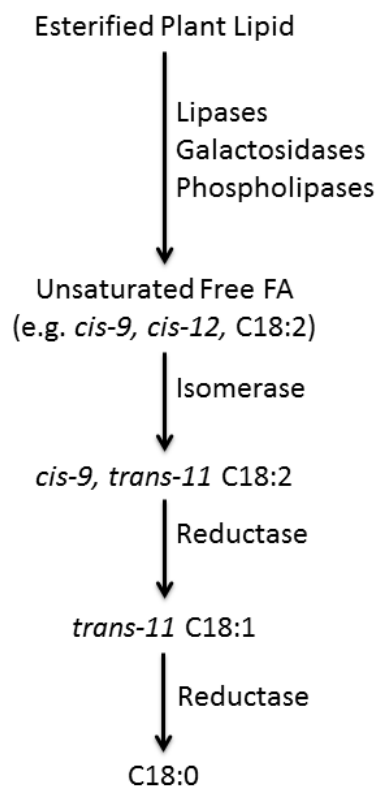
Figure 2.1. Key steps in the conversion of esterified plant lipid to saturated fatty acids by lipolysis and biohydrogenation in ruminal contents.

Adapted from Jenkins (1993).

Feed Management and Biohydrogenation Products

Daily fluctuation in FA production indicates that the rumen undergoes patterns of fermentation over the day. It is a widely held view in ruminant nutrition that rumen microbial populations adapt to dietary changes, but it is yet unclear how consideration of dietary influences on rumen microbial populations could improve nutritional management (Mullins et al., 2013). As

the standard feeding practice for dairy cattle across North America, total mixed ration (TMR) feeding was developed to provide a consistent concentration of fermentable substrate in each meal over the day. However, rumen fermentation is not constant because of variable feed intake levels over the course of a day (Ying et al., 2014). Adapting feed to provide a more appropriate diet relative to fermentation pattern is extremely important. The Harvatine Lab at Penn State has proposed that integration of nutrition with daily rhythms allows for the creation of "circadian-feeding strategies" which are expected to "more precisely meet individual animal requirements while stabilizing rumen fermentation and optimizing nutrient use for milk production in the lactating dairy cow" (Harvatine Personal Correspondence).

Multiple studies have been done in investigate the relationship between dietary factors and abundance of microbial populations and microbial end products produced in the rumen. Identification of the biohydrogenation intermediates in the rumen and their profiles during the daily feeding cycle is an important step in evaluation of the potential to modify absorbed FA profile. It is well documented that milk from cows offered fresh pasture contains higher concentrations of beneficial FA, such as alpha-linolenic acid (C18:3 n-3), conjugated linoleic acid (CLA *cis*-9, *trans*-11), and vaccenic acid (*trans*-11 C18:1) than those offered a total mixed ration or silage and concentrate-based diets (Reviewed by Sun and Gibbs, 2012). Bauman and Griinari (2001) also proposed the 'biohydrogenation theory', which states that diet-induced MFD is caused by an inhibition of mammary lipid synthesis by specific FA that are intermediates in the biohydrogenation of dietary polyunsaturated fatty acid (PUFA). It has been shown that these bioactive intermediates are predominantly produced under certain conditions of altered ruminal fermentation.

Chapter 3

MATERIALS AND METHODS

Animals and Experimental Design

Rumen samples were collected from rumen cannulated lactating dairy cows in a previous experiment conducted by L. Whitney Rottman in the Harvatine Lab at Penn State. A complete description of animals, feed treatments and sample collection can be found in the paper by Rottman et al. (2014a) titled "The effects of feeding rations that differ in NDF and starch concentration within a day on production, feeding behavior, total tract digestibility, and plasma metabolites and hormones in dairy cows." Briefly, nine multiparous Holstein cows (158 ± 48 days in milk, mean ± SD) from the Pennsylvania State University Dairy Herd were randomly assigned to one of three treatments in a 3x3 Latin square design with 23 d periods. Cows were housed in tie stalls and lights were manually controlled with a dark phase from approximately 0000 to 0500 h. Cows were milking in a milking parlor at 0700 and 1700 h.

The three diets used were a control diet (33.1% NDF), a low fiber diet (LF; 29.6% NDF), and a high fiber diet (HF; 34.8% NDF). Forage was substituted for ground corn to change diet NDF, while other ingredients remained constant. The LF and HF diets were balanced to provide the same nutrient composition as the control diet when combined at a ratio of 3 parts of LF and 7 parts of HF on a DM basis (Table 3.1) The control treatment (CON) was fed the control TMR at 0900 h, the high/low treatment (H/L) was fed HF at 70% of daily offering at 0900 h and LF at

30% of daily offering at 2200 h, and the low/high (L/H) treatment was fed LF at 30% of daily

offering at 0900 h and HF at 70% of daily offering at 1300 h (Figure 3.1). All cows were fed at

110% of expected daily intake to allow ad libitum intake. Refused feed was removed before

delivery of new feed at each feeding. Animal care and procedures were approved by the

Pennsylvania State University Institutional Animal Care and Use Committee (#31694).

Rumen Sampling and Analysis

Rumen samples were collected every nine hours on d 15 to 17 to represent every three hours of

the day. Samples were taken from five different locations in the rumen, composited, and whole

digesta stored at -20°C until further analysis. Samples were ground through a 1 mm screen using

a Wiley mill (Aurthur A. Thomas Co., Philadelphia, PA) and analyzed for FA and microbial

population concentration and profile as described below.

Fatty Acid Analysis

Fatty acid concentration and profile of all samples were analyzed according to Jenkins et

al. (2008). Briefly, lipids were direct methylated with a two-step procedure. Digesta was first

transmethylated with sodium methoxide followed by acid methylation with methanolic HCl.

Fatty acid methyl esters were then extracted in hexane, cleaned with activated charcoal, and

quantified by gas chromatography with a flame ionization detector (GC-FID; Agilent 6890A;

Agilent Technologies Inc., Santa Clara, CA) and a capillary column [SP-2560; 100 m × 0.25 mm

(i.d.) with 0.2-μm film thickness; Supelco Inc., Bellefonte, PA]. Fatty acid peaks were identified

in the gas chromatographic analysis using pure methyl ester standards (GLC 780 and 461;

NuChek Prep Inc., Elysian, MN). An equal weight reference standard (GLC 74; NuChek Prep Inc.) was used to determine correction factors for individual FA. Concentration of FA was determined using C13:0 (FFA) and C17:1 (FAME) as internal standards.

Microbial Population Analysis

Microbial population abundance of samples from 6 cows representing a two balanced 3x3 Latin squares was analyzed according to Rico et al. (2013). Briefly, microbial DNA was isolated using a commercial kit (E.Z.N.A.Stool DNA Kit, Omega Bio-tek Inc.). Approximately 200 mg of freeze dried and ground digesta was homogenized for 10 min with 1.25 mL of lysis buffer and 0.2 g of 0.1 mm sterile zirconia beads using a bench-top vortex equipped with a Mo Bio Vortex-Genie 2 adapter (Mo Bio laboratories, Carlsbad, CA). After bead-beating, samples were incubated at 95°C for 10 min with vortexing twice during incubation. Lastly, DNA was purified by column extraction and DNA concentration determined by spectroscopy (NanoDrop ND-1000 Spectohotometer, Nanodrop technologies, Wilmington, DE).

The relative abundance of lactate using bacteria (*Megasphera elsdenii* and *Selenomonas ruminantium*), amyloytic bacteria (*Streptococcus bovis* and *Prevotella bryantii*), fibrolytic bacteria (*Ruminococcus albus* and *Fibrobacter succinogenes*), bacterial involved in biohydrogenation (*Butyrivibrio/Pseudobutyrivibrio* group and *Butytivibrio hungatei* group), total anaerobic fungi, total ciliate protozoa, and total bacteria was determined using quantitative Real-Time PCR (qRT-PCR) and 400 nM of previously validated primers and conditions (Table 3.2). Reactions were conducted in triplicate using a commercial mix (PerfeCTa SYBR Green

SuperMix with ROX, Quanta Biosciences, Gaithersburg, MD) and amplification fluorescence

was measured (Applied Biosystems 7900 HT Fast Real-Time PCR system, Life Technologies,

Grand Island, NY). Primer specificity was evaluated by melting curve analysis and efficiency (E)

was calculated as: $E = 10^{-1/\text{slope of standard curve}}$. Abundance of bacterial species are reported as a

percent of total bacteria as calculated as: $E(UB)^{Ct(UB)} / E(TG)^{Ct(TG)}$ where E(UB) is the efficiency

of the reaction for the universal bacteria primer set, E(TG) is the efficiency of the reaction for the

target gene, and Ct is the average Ct value for each sample (Rico et al, 2013). Abundance of

fungi, protozoa and total bacteria are reported relative to 0000 h of control treatment for each

population.

Statistical Analysis

Time course response variables were analyzed using the MIXED procedure of SAS with

repeated measures (SAS Institute 2003). Fixed effects were treatment, time, and the interaction

of treatment and time. Random effects were cow (sequence), sequence, and period, repeated

variable was time, and subject was cow x period. A reduced model was used when appropriate

based on Bayesian Information Criterion. Preplanned contrasts were CON vs. H/L and H/L vs.

L/H at each time point and the Kenward Rogers adjustment for the denominator degrees of

freedom was used. Significance and tendencies were declared for main effects at $P < 0.05$ and P

< 0.10, respectively, and for interactions at $P < 0.05$ and $P < 0.10$, respectively.

Table 3.1. Ingredient and nutrient composition of experimental diets.

	Diet[1]		
	CON	HF	LF
Dietary Ingredients, % DM			
Corn silage[2]	38.8	41.7	32.1
Ground corn	19.3	15.1	29.1
Alfalfa haylage[3]	12.6	13.5	10.4
Canola meal	9.6	9.6	9.6
Cracked roasted soybeans	7.2	7.2	7.2
Grass hay & straw[4]	4.1	4.5	3.4
Cookie meal	3.3	3.3	3.3
Vit. & min. mix[5]	3.2	3.2	3.2
Cottonseed hulls	1.41	1.51	1.16
Non-protein N[6]	0.42	0.42	0.42
Nutrients Composition, % DM			
OM	93.8	93.3	93.7
NDF	33.3	34.8	29.6
Starch	28.6	27.2	31.9
CP	15.9	16.0	15.7
Fatty acids			
Total	3.9	3.8	4.1
C16:0	0.56	0.58	0.55
C18:2 n6	1.74	1.69	1.87
C18:1 n9	0.92	0.90	0.97
C18:3 n3	0.26	0.25	0.26

Adapted from Rottman et al. (2014a).
[1]CON = Control, HF = High forage diet, LF = Low Forage diet.
[2]Contained 39.6% NDF, 37.0% starch, and 7.0% CP on a DM basis.
[3]Contained 51.1% NDF and 21.0% CP on a DM basis.
[4]Mixed grass hay and wheat straw mixture. Contained 74.7% NDF and 6.4% CP on a DM basis.
[5]Vitamin and mineral mix contained: 11% CP, 18% NDF, 5.1% fat, 14% Ca, 0.35% P, 4.6% Mg, 0.42% K, 0.3% S, 1,071 ppm Mn, 357 ppm Cu, 1,085 ppm Zn, 6.66 ppm Se, 6.4% salt (DM basis), 262,101 IU vitamin A, 65,421 IU/kg vitamin D, and 1,972 IU/kg vitamin E (DM basis).
[6]Optigen, Alltech Inc. Nicholasville, KY (256% CP, DM basis).
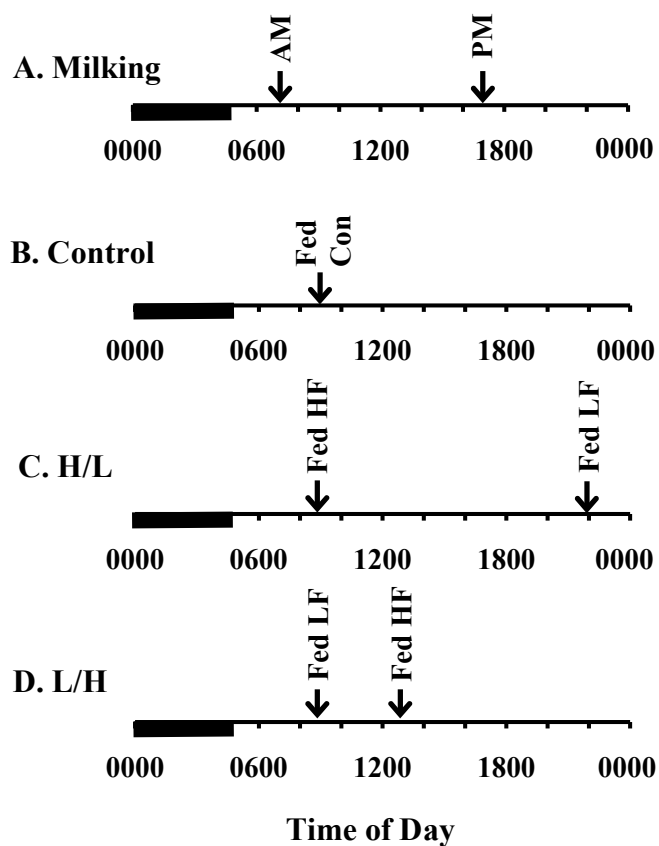
Figure 3.1. Timing of feeding and milking of three treatments tested.

Adapted from Rottman et al. (2014a).

All cows were milked at 0700 and 1700 h each day. Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Dark phase is shown by the black bar.

Table 3.2. List of primers used in real-time assay.

Target	5' – sequence[1]	Ta[2]	Reference[3]
Megasphaera elsdenii	f-GACCGAAACTGCGATGCTAGA	60	1
	r-CGCCTCAGCGTCAGTTGTC		
Ruminococcus albus	f-CCCTAAAAGCAGTCTTAGTTCG	61	2
	r-CCTCCTTGCGGTTAGAACA		
Selenomonas ruminantium group	f-CAATAAGCATTCCGCCTGGG	61	3
	r-TTCACTCAATGTCAAGCCCTGG		
Fibrobacter succinogenes	f-GCGGGTAGCAAACAGGATTAGA	60	3
	r-CCCCCGGACACCCAGTAT		
Streptococcus bovis	f-TTCCTAGAGATAGGAAGTTTCTTCGG	60	3
	r-ATGATGGCAACTAACAATAGGGGT		
Prevotella bryantii	f-AGCGCAGGCCGTTTGG	61	3
	r-GCTTCCTGTGCACTCAAGTCTGAC		
B. fibrisolvens/Pseudobutyrivibrio	f-GCCTCAGCGTCAGTAATCG	65	4
	r-GGAGCGTAGGCGGTTTTAC		
Butytivibrio hungatei	f-AGGGTAATGCCTGTAGCTC	55	4
	r-TCACCCTCGCGGGAT		
Fungi	f-GAGGAAGTAAAAGTCGTAACAAGGTTTC	60	5
	r-CAAATTCACAAAGGGTAGGATGATT		
Ciliate protozoa	f-GCTTTCGWTGGTAGTGTATT	54	6
	r-CTTGCCCTCYAATCGTWCT		
Total Bacteria	f-CGGCAACGAGCGCAACCC	60	5
	r-CCATTGTAGCACGTGTGTAGCC		

[1]Selected forward (f) and reverse (r) primers for validated for genes encoding 16S ribosomal RNA (bacterial species and fungi), and for the 18S ribosomal RNA gene (protozoa).
[2]Annealing temperature.
[3]Source of primers: Denman and McSweeney, 2006 (1), Koike and Kobayashi, 2001 (2), Ouwerkerk et al., 2002 (3), Shingfield et al., 2012 (4), Stevenson and Weimer, 2007 (5), Sylvester et al., 2004 (6).

Chapter 4

RESULTS

Rumen Fatty Acid Profile

Rumen digesta FA extraction, methylation, and quantification were successful in determining ruminal FA composition in 215 of 216 samples. The sample eliminated failed to result in a quantifiable concentration of methylated FA, as shown by a total peak area below 500 units (normally 1000 to 2000 units). Individual FA data points were considered outliers and eliminated from the remaining 215 samples during statistical analysis if their Studentized residuals were outside of $\pm$ 3.0 (equivalent to $\pm$ 3.0 standard deviations).

Rumen digesta FA profile is summarized in Table 4.1. All FA were quantified as a percent of total rumen FA and showed a significant effect of time except for C14:0 and *cis-9, trans-*11 CLA. A main effect of treatment was not observed except for *cis-*11 C18:1 and C18:3. However, there was a treatment by time interaction on *cis-*9 C18:1, *cis-*12 C18:1, and C20:1 and a tendency for a treatment by time effect on C17:0, *trans-*10 C18:1, *trans-*11 C18:1, and C20:0.

Rumen FA concentrations of 14 to 16 carbon FA (C14:0, C15:0, C16:0, and C16:1) as a percent of total FA throughout the day for all three treatments are shown in Figure 4.1. Significant differences in preplanned contrasts are noted. There was a tendency for C14:0 (Panel A) in HL to be 7% lower than control at 2100 h. Additionally, C15:0 (Panel B) tended to be 6%

higher in HL compared to control concentration at 1800 h and almost 6% lower in HL compared to LH at 0000 h and was 10% lower in HL at 0300 h. There was no effect of treatment on C16:0 at any time point (Panel C). However, C16:1 was 9% higher in HL compared to LH at 2100 h (Panel D).

Rumen 17 carbon FA are shown in Figure 4.2 and 18 carbon FA are shown within Figure 4.2 to 4.4. Specifically, C18:0, *trans*-5 C18:1, and t*rans* 6-8 C18:1 are shown in Figure 4.2. Rumen C17:0 tended to be 3% lower in HL compared to control at 0000 h and was approximately 5% lower in HL compared to LH at 0000 and 0300 h and was 4% higher in HL compared to LH at 2100 h (Figure 4.2 Panel A). Rumen C18:0 was 8% lower in HL compared to control at 0000 h and tended to be 2% lower at 0300 h (Figure 4.2 Panel B). Rumen C18:0 was also nearly 9% higher in HL compared to LH at 0300 h and tended to be higher at 0000 h (Figure 4.2 Panel C). *Trans-5* C18:1 tended to be 12% lower in HL compared to LH at 0000 h. Lastly, *trans-6-8* C18:1 was 8% lower in HL compared to control at 2100 h and HL was 9% lower than LH at 0300 h (Figure 4.2 Panel D).

Rumen FA concentrations of 18 carbon FA with *trans-* bonds as a percent of total FA throughout the day are shown in Figure 4.3 including *trans*-9 C18:1, *trans*-10 C18:1, *trans*-11 C18:1, and *trans*-12 C18:1. There was a tendency for *trans*-9 C18:1 (Panel A) in HL to be 7% lower than control at 2100 h . Additionally, at 0300 h rumen *trans*-9 C18:1 was 8% higher in HL compared to LH. Rumen *trans*-10 C18:1 (Panel B) was 13% higher in HL at 0300 h compared to both control and LH and was 11% and 10% higher in HL at 2100 h in HL compared to control and LH respectively. Further, rumen *trans*-11 C 18:1 was 7% lower in HL compared to LH at

0600 h (Panel C). There was no effects of treatment on *trans*-12 C18:1 at any time point (Panel D).

Rumen FA concentrations of 18 carbon FA with *cis*-bonds (*cis*-9 C18:1, *cis*-10 C18:1, and *cis*-11 C18:1) as a percent of total FA throughout the day are shown in Figure 4.4. There was a tendency for *cis*-9 C18:1 (Panel A) at 0000 h in HL to be 35% higher in HL compared to control and 16% higher compared to LH. At 0300 h, rumen *cis*-9 C18:1 was 22% higher in HL compared to control and 33% higher compared to LH. In addition, *cis-9* C18:1 was 23% lower in HL compared to LH at 0900 h. Rumen *cis*-10 C18:1 (Panel B) was 13% higher in HL compared to LH at 0000 h and at 0300 h tended to be 12% higher in HL compared to control as well as 22% higher in HL compared to LH. Lastly, compared to control, c*is*-12 C18:1 was 22% higher in HL at 0000 h and 20% higher at 0300 h (Panel C). *Cis*-11 C18:1 also was 27% higher in HL compared to LH at 0000 h.

Rumen FA concentrations of polyunsaturated 18 carbon FA and 20 carbon FA (C18:2, C18:3, C20:0, and C20:1) as a percent of total FA over the day are shown in Figure 4.5. There was a tendency for C18:2 (Panel A) in HL to be 45% higher compared to LH at 0300 h and was 35% higher in HL compared to control at 0000 h. There was no effect of treatment on C18:2 at any time point (Panel B). At 0000 h, C20:0 tended to be 3% lower in HL compared to LH as well as was an almost 5% lower in HL compared to control. Rumen C20:0 tended be 4% lower in HL compared to LH at 0900 h and almost 3% higher in HL compared to LH at 1200 h. Finally, C20:1 was 27% and 21% higher in HL compared to control at 0300 and 0900 h, respectively (Panel D).

Rumen FA concentrations of conjugated linoleic acids (*cis*-9*, trans*-11 CLA and *trans*-10, *cis*-12 CLA) as a percent of total FA throughout the day are shown in Figure 4.6. There was a tendency for *cis*-9, *trans*-11 CLA (Panel A) in HL to be 39% higher than LH at 0600 h and 47% higher in HL compared to control at this same time point. *Cis*-9 *trans*-11 CLA also was 33% higher in HL at 1200 h and 30% higher in HL at 2100 h compared to control. Lastly, *trans*-10, *cis*-12 CLA was 28% higher in HL compared to control at 1800 h (Panel B).

Rumen Microbial Population Profile

Rumen digesta DNA extraction, purification, and quantification were successful in determining ruminal microbial population composition in 143 of 144 samples. Individual microbial DNA concentration data points were eliminated from the remaining 143 samples during statistical analysis if they were outside of $\pm$ 3.0 standard deviations from the mean.

Rumen digesta microbial population profile is summarized in Table 4.2. All bacterial species were quantified as a percent of total bacteria. Total fungi and ciliate protozoa were quantified in relation to total bacteria as well as for relative abundance. Total bacteria was quantified for relative abundance. Most microbial populations as percent total bacteria showed a significant effect of time except for *M. elsdenii, B. fibrisolvens/Pseudobutyrivibrio,* and total fungi, as well as *B. hungatei,* which only showed a tendency for an effect of time. However, only two of these, *F. succinogenes* and total ciliate protozoa, showed an effect of treatment and only

one population, *R. albus,* had a significant treatment by time interaction. Finally, significant treatment and time effects, but no treatment by time interaction, were observed for ciliate protozoa relative abundance.

Rumen bacteria concentrations as a percent of total bacteria throughout the day for all three treatments are shown in Figure 4.7to Figure 4.9. Specifically, *Megasphera elsdenii*, *Ruminococcus albus*, *Selenomonas ruminatium* group, and *Fibrobacter succinogenes* are shown in Figure 4.7. Significant differences in preplanned contrasts are noted. *M. elsdenii* (Panel A) was 275% lower in HL concentration compared to LH at 0900 h. In contrast, *R. albus* was only 57% lower in HL compared to LH at 0900 h as well as 50% lower in HL compared to control at 0300 h (Panel B). *S. ruminatium* group (Panel C) was over 200% higher in HL compared to control at 0900 h. Finally, *F. succinogenes* was 66% lower in HL compared to LH at 0600 h (Panel D).

Streptococcus bovis, Prevotella bryantii, B. fibrisolvens/Psuedobutyrivibrio, and *Butytivibrio hungatei* as a percent of total bacteria are shown in Figure 4.8. *S. bovis* was over 1000% higher in HL compared to control at 0900 h (Panel A). There was a tendency for *P. bryantii* (Panel B) to be almost 9% higher in HL than LH at 1200 h and over 200% higher in HL compared to control at this time point. At 1500 h, *P. bryantii* also was 78% and over 200% higher in HL compared to control and LH, respectively. *B. fibrisolvens/Psuedobutyrivibrio* (Panel C) was almost 250% higher in HL compared to control as well as 83% higher compared to LH at 0900 h. Additionally, *B. hungatei* was almost 500% higher in HL compared to control at 0900 h.

Rumen abundance of ciliate protozoa and fungi relative to total bacteria throughout the day for all three treatments are shown in Figure 4.9. Total anaerobic fungi (Panel A) were over 350% higher in HL compared to LH and 67% higher in HL compared to control at 0900 h. There was a tendency for ciliate protozoa to be 51% and 47% lower in HL than control at both 0600 h and 0900 h, respectively (Panel B). Protozoa were over 100% and 60% lower in HL compared to control at 0300 h and 1500 h, respectively. Finally, HL was 63% lower compared to LH at 0600 h.

Rumen ciliate protozoa, anaerobic fungi, and total bacteria abundance relative to control at 0700 h is shown in **Error! Reference source not found.**. There was no effect of treatment on anaerobic fungi relative abundance at any time points (Panel A). There was a tendency for ciliate protozoa relative abundance (Panel B) at 0000 h in HL to be almost 40% lower compared to control, and was 95% lower in HL compared to control at 0300 h. There was a tendency for ciliate protozoa to be 70% lower in HL compared to LH at 0300 h and almost 90% lower HL compared to LH at 0600 h. Lastly, total bacteria relative abundance (Panel C) tended to be 27% lower at 0000 h in HL compared to LH.

Table 4.1. The effect of feeding a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on rumen digesta fatty acid profile.

FA, % of FA	Treatment Means [1]				P values [2]			H/L vs. CON	H/L vs. L/H
	CON	H/L	L/H	SEM	*Trt*	*Time*	*Trt×Time*		
C14:0	0.96	0.94	0.94	0.08	0.48	0.24	0.83	0.32	0.92
C15:0	0.65	0.65	0.66	0.02	0.31	<0.001	0.16	0.67	0.29
C16:0	15.00	15.00	14.99	0.10	0.99	<0.001	0.96	1.00	0.89
C16:1	0.30	0.30	0.30	0.01	0.47	<0.001	0.31	0.22	0.56
C17:0	0.38	0.38	0.38	0.02	0.45	<0.001	0.10	0.81	0.23
C18:0	45.01	44.23	44.73	0.98	0.40	<0.001	0.23	0.18	0.39
trans-5 C18:1	0.07	0.07	0.07	0.003	0.47	<0.001	0.62	0.35	0.25
trans-6–8 C18:1	0.64	0.63	0.62	0.03	0.44	<0.001	0.32	0.70	0.39
trans-9 C18:1	0.41	0.41	0.41	0.02	0.61	<0.001	0.41	0.92	0.42
trans-10 C18:1	0.89	0.90	0.90	0.04	0.90	<0.001	0.14	0.65	0.88
trans-11 C18:1	3.00	3.02	3.01	0.15	0.97	<0.001	0.14	0.81	0.99
trans-12 C18:1	0.88	0.87	0.86	0.03	0.37	<0.001	0.47	0.53	0.43
cis-9 C18:1	7.18	7.51	7.37	0.32	0.34	<0.001	<0.01	0.15	0.52
cis-11 C18:1	1.22	1.28	1.22	0.05	0.07	<0.001	0.51	0.05	0.04
cis-12 C18:1	0.91	0.93	0.90	0.05	0.65	<0.001	0.07	0.61	0.36
C18:2	4.90	5.44	5.13	0.25	0.15	<0.001	0.57	0.05	0.27
C18:3	0.54	0.62	0.60	0.04	0.08	<0.001	0.81	0.03	0.61
C20:0	0.66	0.65	0.66	0.01	0.19	<0.001	0.10	0.08	0.17
C20:1	0.11	0.11	0.11	0.004	0.55	<0.001	0.03	0.28	0.68
CLA [3]	0.12	0.15	0.12	0.01	0.24	<0.001	0.34	0.12	0.19
cis-9, trans-11	0.09	0.10	0.10	0.01	0.20	0.50	0.63	0.09	0.71
trans-10, cis-12	5.86	5.90	5.87	0.23	0.94	<0.01	0.16	0.75	0.78

[1]Least square means. The control (CON) fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h.
[2]Significance of preplanned contrasts (*H/L vs. CON* and *H/L vs. L/H*) and the main effect of treatment (*Trt)*, time (time), and the interaction of treatment and time (*Trt×Time*).
[3]Conjugated linoleic acid.

Table 4.2. The effect of feeding a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on rumen digesta microbial population profile.

Microbial population	Treatment Means [1]				P values [2]				H/L vs. CON	H/L vs. L/H
	CON	H/L	L/H	SEM	Trt	Time	Trt× Time	Block		
% of total bacteria [3]										
Megasphaera elsdenii	0.01	0.01	0.01	0.002	0.17	0.15	0.24	0.003	0.82	0.09
Ruminococcus albus	0.19	0.17	0.19	0.02	0.31	<0.001	0.06	0.41	0.15	0.27
Selenomonas ruminantium group	1.90	2.21	2.19	0.26	0.45	0.02	0.91	0.65	0.24	0.85
Fibrobacter succinogenes	1.53	1.47	1.72	0.14	0.05	<0.001	0.69	0.06	0.53	0.02
Streptococcus bovis	0.02	0.03	0.04	0.02	0.55	0.05	0.90	0.03	0.39	0.87
Prevotella bryantii	0.20	0.23	0.20	0.036	0.45	0.08	0.42	0.001	0.35	0.23
B. fibrisolvens/ Pseudobutyrivibrio	2.47	2.97	2.64	0.38	0.48	0.54	0.83	0.01	0.23	0.44
Butytivibrio hungatei	0.31	0.45	0.40	0.09	0.44	0.14	0.89	0.002	0.21	0.67
Fungi	1.38	1.77	1.58	0.21	0.29	0.53	0.63	0.003	0.12	0.45
Ciliate Protozoa	10.00	6.69	7.85	1.31	0.01	0.09	0.68	0.01	0.001	0.22
Relative abundance [4]										
Fungi	0.93	1.05	1.09	0.40	0.73	0.61	0.87	---	0.51	0.56
Ciliate Protozoa	1.31	0.94	1.16	0.31	0.04	<0.01	0.39	---	0.32	0.35
Total Bacteria	1.14	0.98	1.07	0.31	0.47	0.5	0.56	---	0.50	0.39

[1]Least square means. The control (CON) fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h.
[2]Significance of preplanned contrasts (*H/L vs. CON* and *H/L vs. L/H*) and the main effect of treatment (*Trt)*, time (time), and the interaction of treatment and time (*Trt×Time*).
[3]Relative abundance (% of target gene relative to total bacterial 16S rDNA).
[4]Data standardized relative to d 0 of control (1.0).
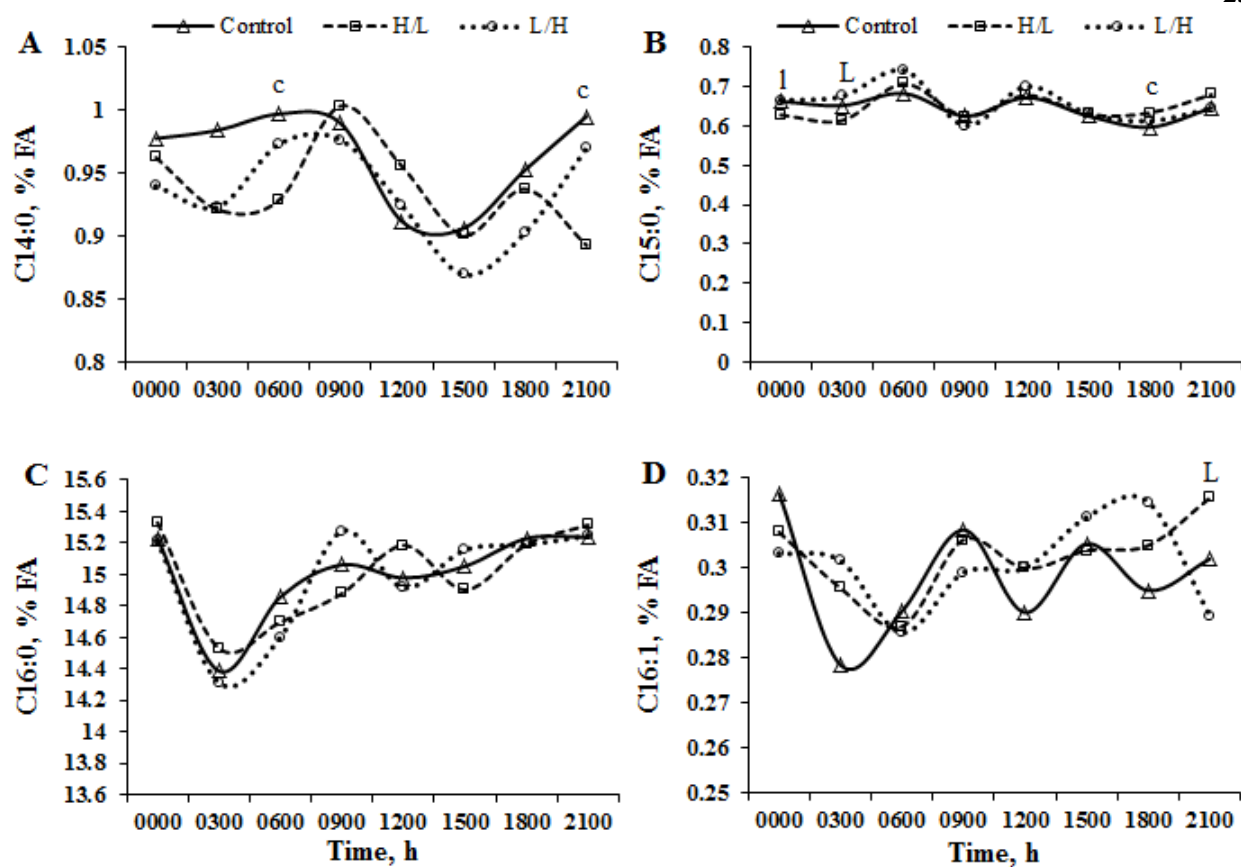
Figure 4.1. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on fatty acid concentration profile including C14:0 (A) C15:0 (B), C16:0 (C), and C16:1 (D).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.1. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and $c = P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and $l = P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
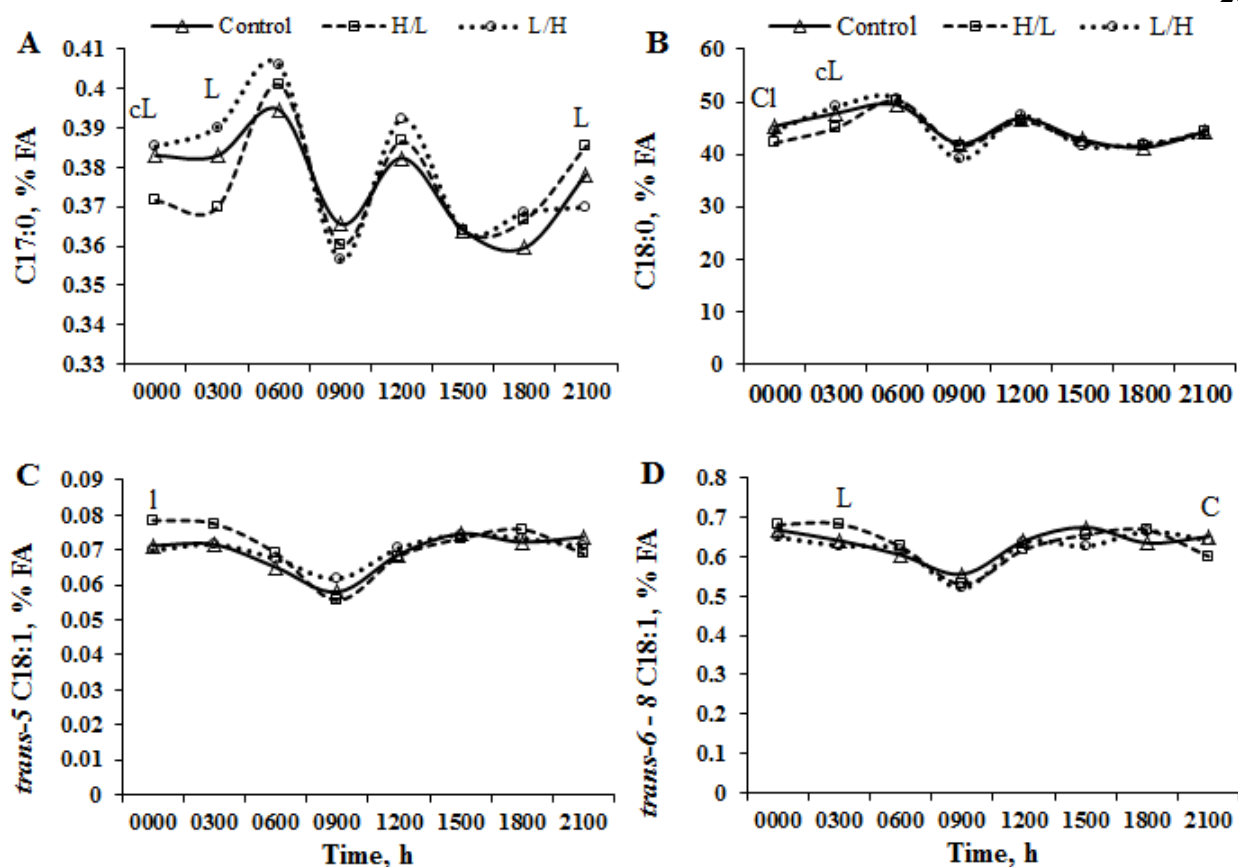
Figure 4.2. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on fatty acid concentration profile including C17:0 (A) C18:0 (B), *trans-5* C18:1 (C), and *trans-6 – 8* C18:1 (D).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.1. Table 4.1 Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and c $= P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and l $= P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
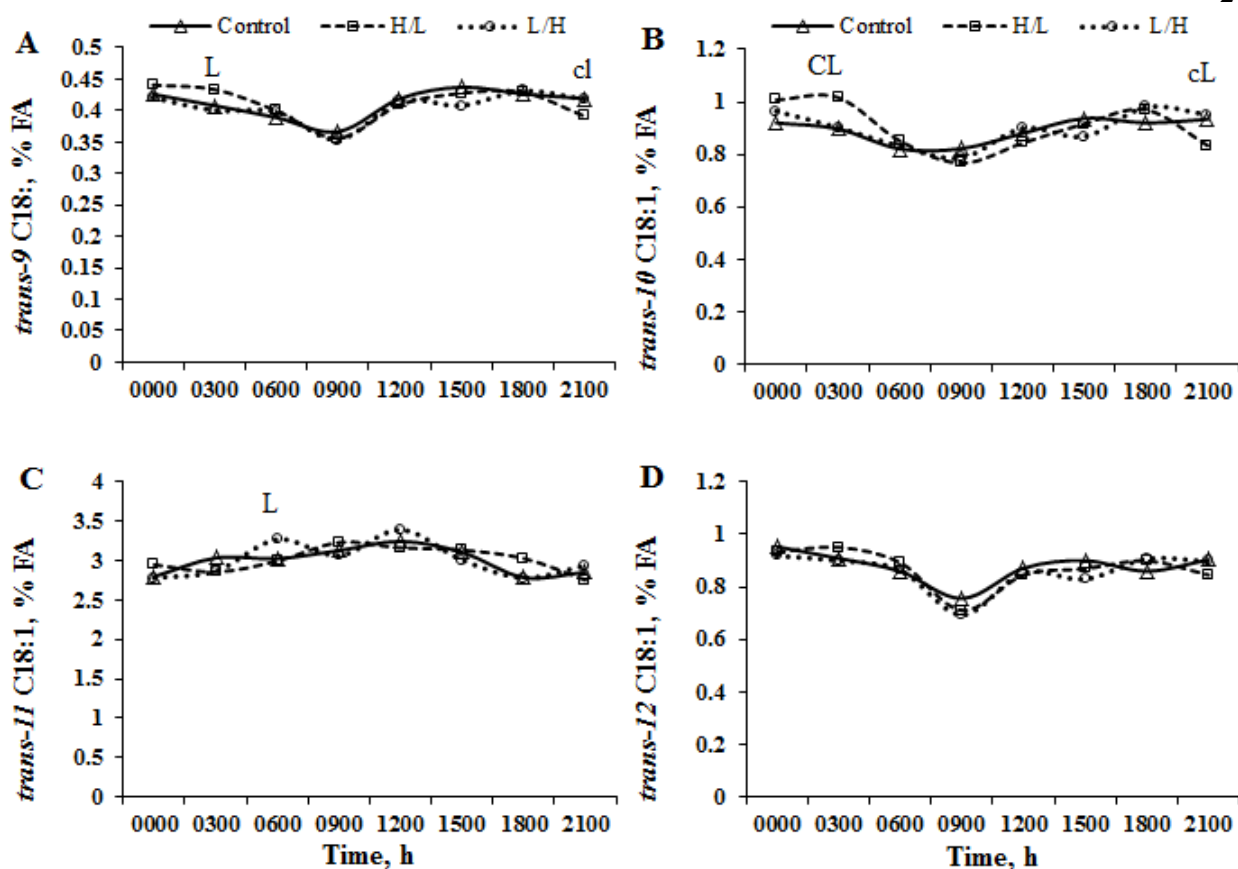
Figure 4.3. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on fatty acid concentration profile including *trans-9* C18:1 (A) *trans-10* C18:1 (B), *trans-11* C18:1 (C), and *trans-12* C18:1 (D).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.1. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and c $= P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and l $= P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
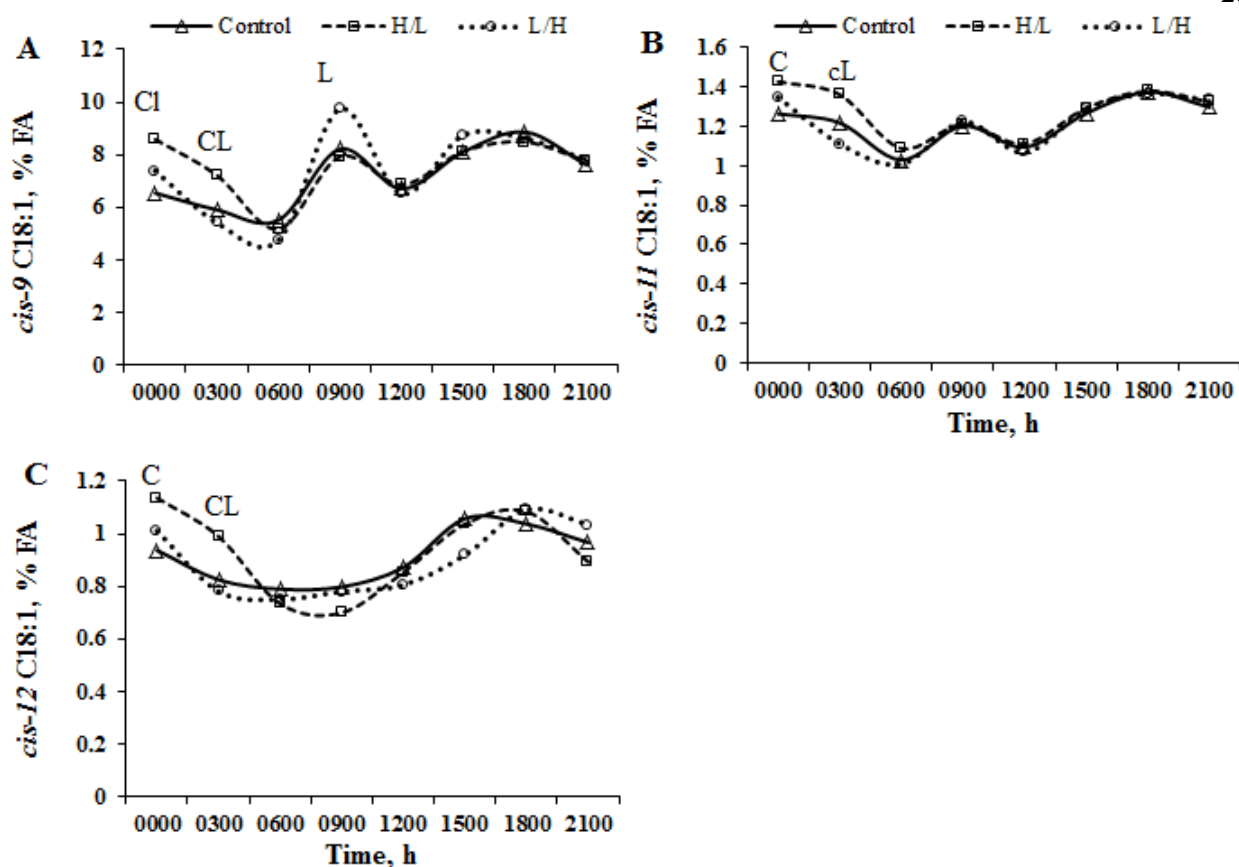
Figure 4.4. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on fatty acid concentration profile including *cis-9* C18:1 (A) *cis-11* C18:1 (B), and *cis-12* C18:1 (C).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.1. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and $c = P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and $l = P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
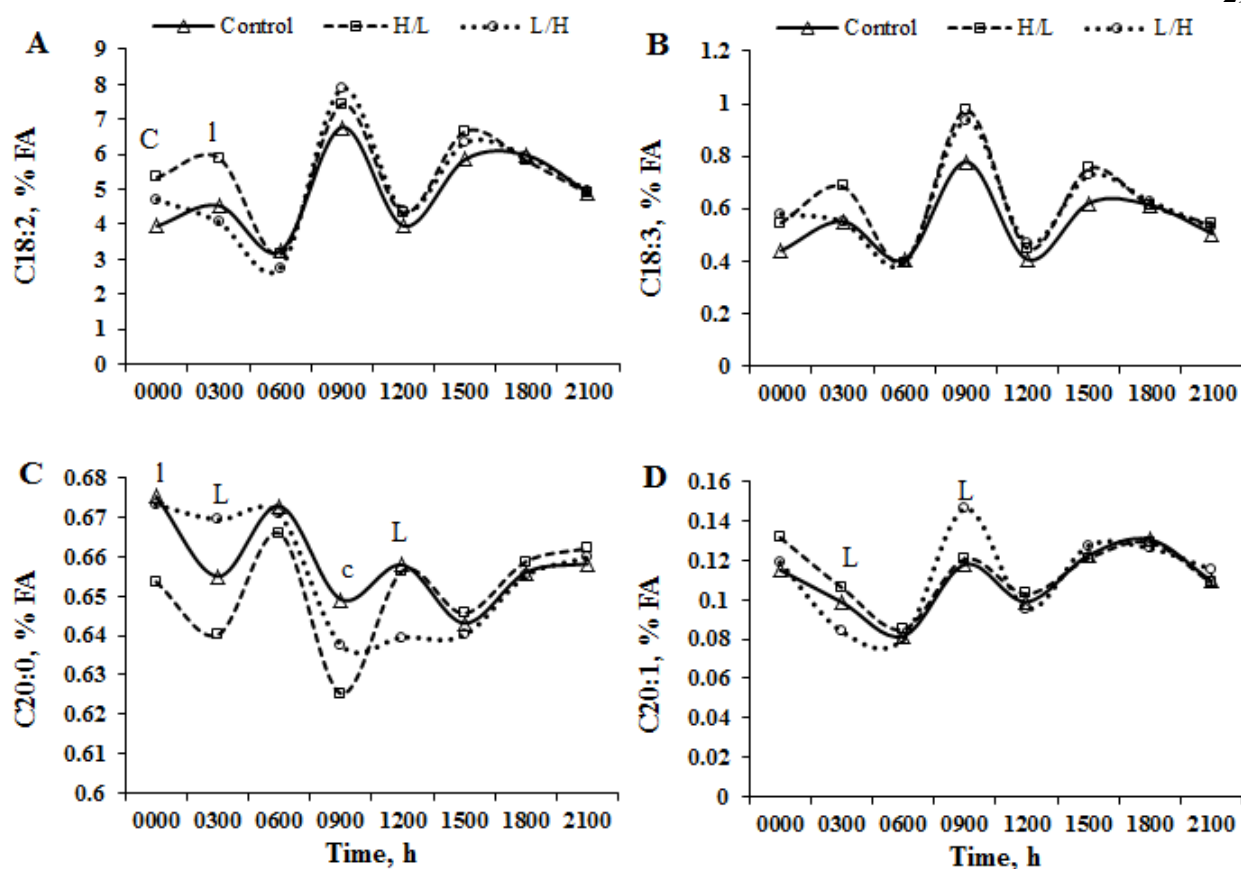
Figure 4.5. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on fatty acid concentration profile including C18:2 (A) C18:3 (B), C20:0 (C), and C20:1 (D).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.1. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and $c = P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and $l = P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
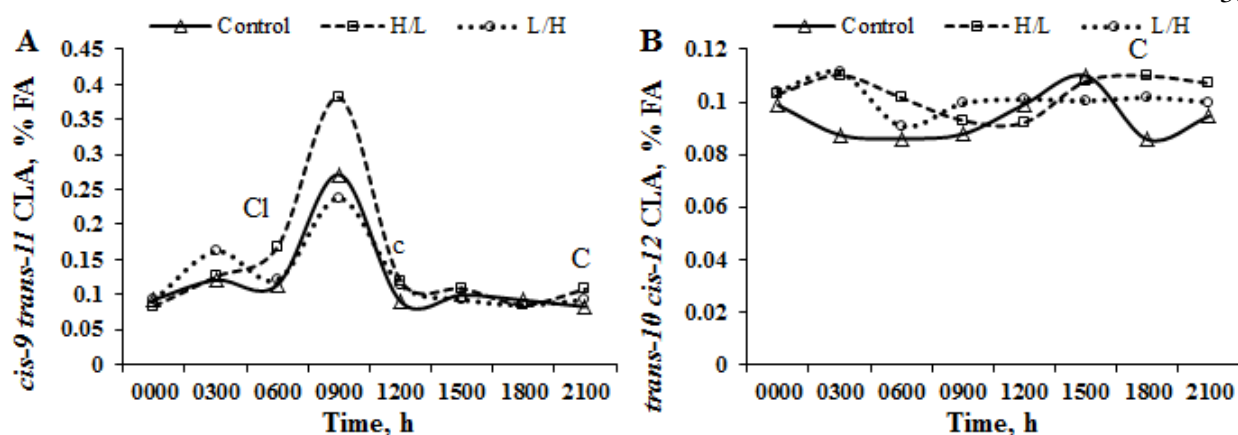
Figure 4.6. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on fatty acid concentration profile including *cis-9 trans-11* CLA (A) and *trans-10 cis-12* CLA (B).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.1. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and c = $P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and l = $P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
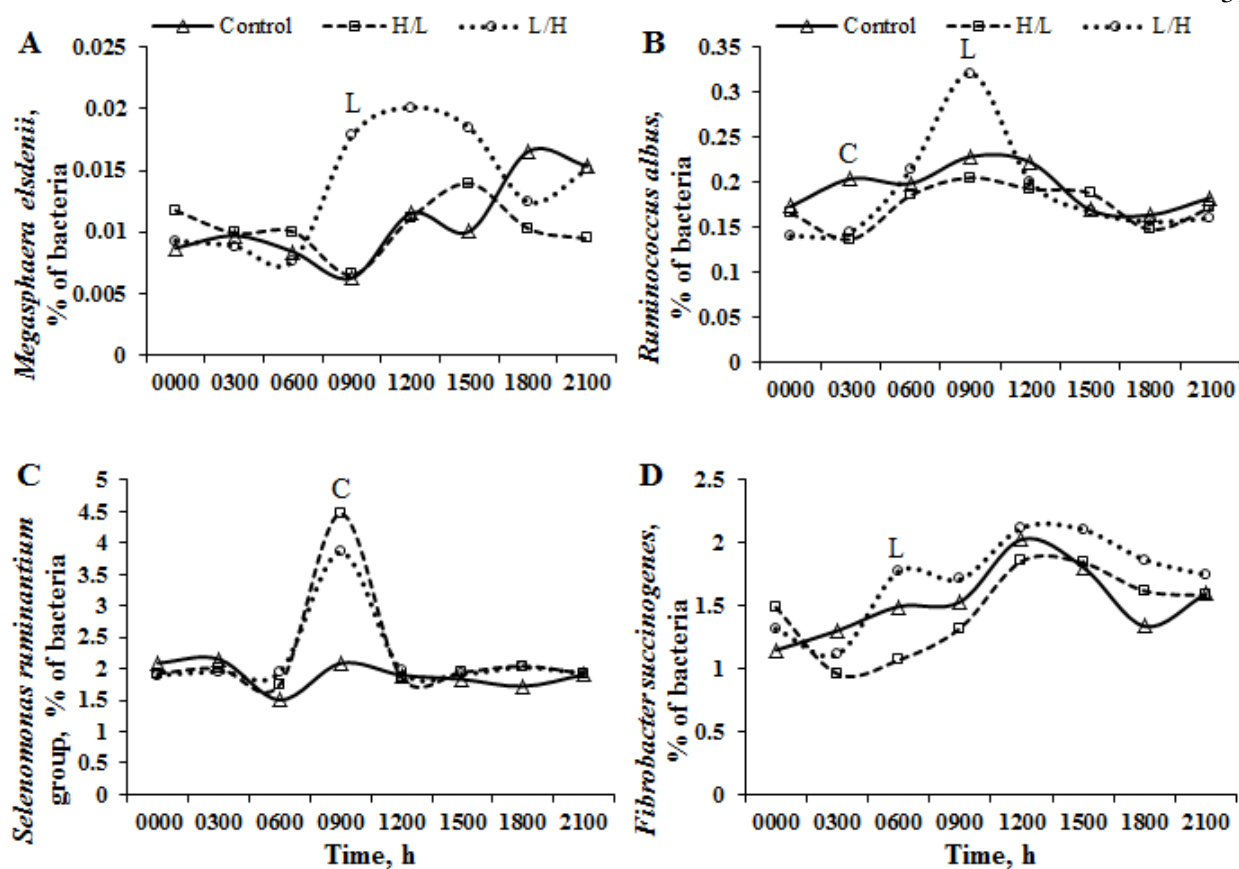
Figure 4.7. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on rumen digesta microbial population profile in relation to total percent bacteria including *Megasphaera elsdenii* (A) *Ruminococcus albus* (B), *Selenomonas ruminatium* (C), and *Fibrobacter succinogenes* (D).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.2. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and c $= P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and l $= P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.

Figure 4.8. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on rumen digesta microbial population profile in relation to total percent bacteria including *Streptococcus bovis* (A) *Prevotella byrantii* (B), *B. fibrisolvens/Psuedobutyrivibrio* (C), and *Butytivibrio hungatei* (D).
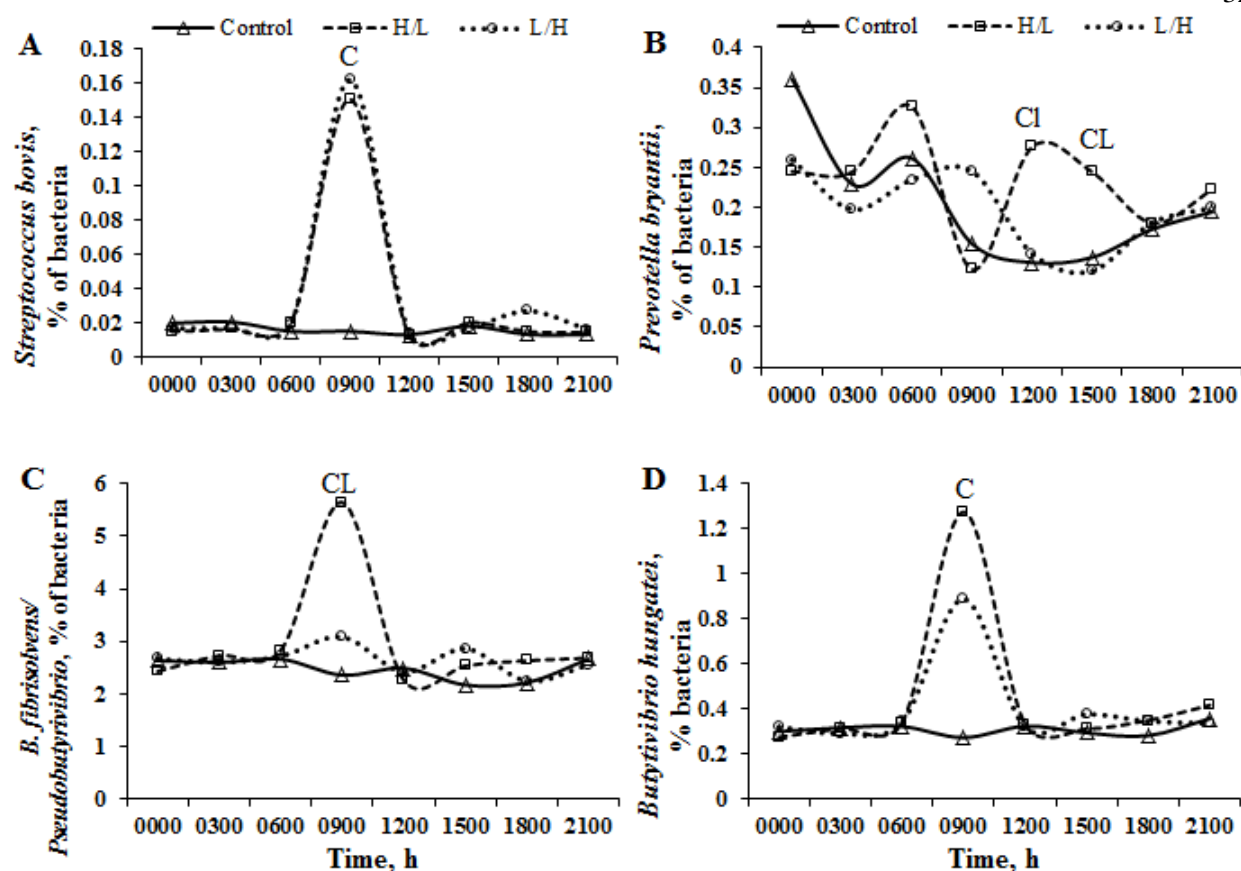
Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.2. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and $c = P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and $l = P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.

Figure 4.9. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on rumen digesta microbial population profile in relation to total percent bacteria including Fungi(A) and Ciliate Prototozoa (B).
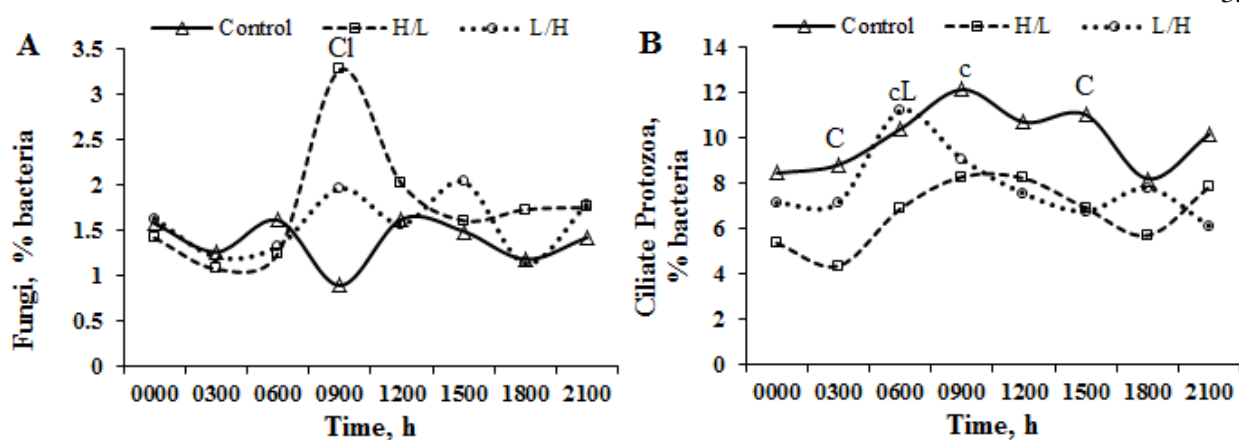
Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.2. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and $c = P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and $l = P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.
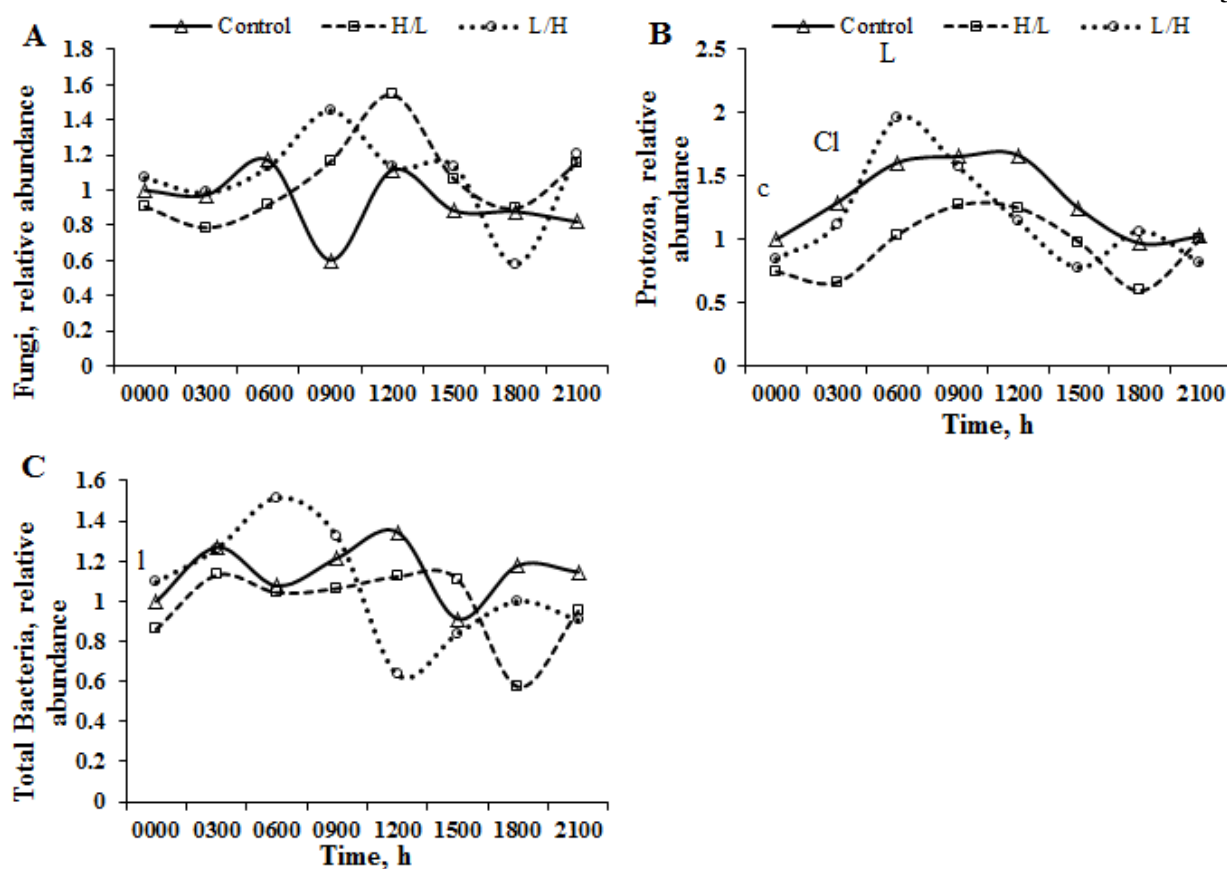
Figure 4.10. The effect of a single TMR or feeding two rations that differ in their fiber and starch concentration at different times of the day on rumen digesta microbial population profile relative to abundance of Control time 0000 h including Fungi(A) Ciliate Protozoa (B), and Total Bacteria (C).

Control was fed a TMR once per day at 0900 h, H/L fed a high forage ration at 70% of daily offering at 0900 h and a low forage ration at 30% of daily offering at 2200 h, and L/H fed a low forage ration at 30% of daily offering at 0900 h and a high forage diet at 70% of daily offering at 1300 h. Significance of treatment, time, and their interaction and SEM reported in Table 4.2. Preplanned contrasts at each time point are shown [control vs. H/L ($C = P < 0.05$ and c $= P < 0.1$)]; H/L vs. L/H ($L = P < 0.05$ and l $= P < 0.1$)]. Dark phase was approximately 0000 to 0500 h.

Chapter 5

DISCUSSION

Rumen Fatty Acid Profile

Feed derived FA are predominantly *cis*-9 C18:1, C18:2, and C18:3. The LF diet was slightly higher in *cis*-9 C18:1 and C18:2, as concentrate feeds are a good source for these FA, while forages are higher in C18:3. Rumen *cis*-9 C18:1 FA concentration was over 32% higher in H/L compared to control from 0000 through 0300 h due to the large amount of the low forage diet consumed after the evening feeding (Ying et al., 2014). Similarly, *cis-9* C18:1 was 22% higher in L/H than H/L at 0900 h which followed morning feeding of the low forage diet. Following a similar mechanism, C18:2 was 35% higher in H/L compared to control at 0000 h.

Dietary unsaturated FA are biohydrogenated in the rumen to form a number of different *trans*- intermediates before producing C18:0 as the final biohydrogenation product. In short, the normal pathway of biohydrogenation includes the isomerization of *cis*-9 *cis*-12 C18:2 to *cis*-9 *trans*-11 CLA, followed by hydrogenation to *trans*-11 C18:1 and final hydrogenation to C18:0. This pathway predominates with high fiber diets. However, alternate pathways exist for biohydrogenation of unsaturated FA, including isomerization of *cis*-9 *cis*-12 C18:2 to *trans*-10 *cis*-12 CLA, followed by hydrogenation to either *trans*-10 C18:1 or *cis*-12 C18:1 and final hydrogenation to C18:0. The alternate biohydrogenation pathway occurs with higher starch diets and lower rumen pH. Generally, *trans*-10 C18:1 and *cis*-12 C18:1 reached a nadir between 0600

and 0900 h and tended to increase compared to control at 2100 h immediately after evening feedings of the low forage diet. *Trans*-11 C18:1 peaked between 0900 and 1500 h, which was around feeding of high forage diet. Loor et al. (2004) saw these same effects in all dietary treatments, where *trans*-10 *cis*-12 CLA was nearly undetectable at 0900 h regardless of diet and *trans*-10 C18:1 peaked later in the day at 1700 h. Surprisingly, *cis*-9 *trans*-11 CLA was the most predominant FA regardless of diet, and *trans*-11 C18:1 did not differ with diet or time (Loor et al., 2004).

Lastly, C15:0 and C17:0 are odd-chain FA originating from microbial synthesis and C17:0 is correlated with total microbial flow and higher fiber diets (Vlaeminck et al., 2006). The tendency for a treatment by time interaction for C17:0 ($P = 0.10$), showed over a 4% increase in C17:0 in H/L compared to L/H at 2100 h, but approximately a 3% decrease in C17:0 in H/L compared to control at 0000 and 0300 h. This decrease in C17:0 indicates a reduced fibrolytic bacterial growth during this phase of the day for the H/L treatment and was verified by both fibrolytic bacteria quantified in this study.

Changes in the absorption of specific FA over the day is also expected, but was not determined in the current experiment because this would require duodenally cannulated cows. Milk FA profile provides an indication of the change in FA absorbed over the day, although at a much lower resolution. Rottman et al. (2014b) milked cows 4x/d and observed little difference in milk FA profile, including the *trans* FA. Additionally, Ma et al. (2015) also reported little change in milk fat *trans*- isomers and *trans*- isomer inhibition of milk fat synthesis in cows milked 3x/d during milk fat depression.

Rumen Microbial Population Profile

The abundance of specific microbial populations is expected to be a result of the rumen environment and the type of substrate available for fermentation. Fibrolytic bacteria have the capacity to synthesize cellulolytic and hemicellulolytic enzymes and therefore are highly correlated with high fiber diets. Both *Ruminococcus albus* and *Fibrobacter succinogenes* saw a reduced abundance from 0000 to 0300 h of the H/L treatment compared to control, which corresponds to the low fiber diet fed at 2100 h. Both bacterial species also showed a spike in concentration between 600 to 1200 h after high fiber feeding of the H/L diet at 0700 h. These changes are expected to be driven by the amount of digestible fiber available for fermentation at these times.

The amylolytic process, or amylolysis, is the process of converting starch into sugar by the action of acids or enzymes. Therefore, amylolytic bacteria are predominately found in larger quantities with low fiber, or high concentrate, diets as well as low pH. More specifically, some amylolytic bacterial species produce lactic acid, such as *Streptococcus bovis*. These bacteria have the ability to grow explosively when large amounts of starch or sugars are present in the rumen. *S. bovis* saw a dramatic increase in microbial concentration between 600 and 1200 h after morning feeding at 0700 h in both HL and LH treatments. It was thought that *S. bovis* populations may be maintained throughout the night after L diet was fed at 2100 h of HL treatment and therefore multiply faster at feeding time, however, this was not shown in the data.

Therefore, although H feed of HL diet contained less concentrate than the L feed of the LH diet

at this time, a large intake of feed in general may still result in a large conversion of any starch

and sugars in the diet to be broken down. *Prevotella bryantii* was also found to spike after each

feeding time of both HL and LH treatments because of the newly available concentrate entering

the rumen.

Some amylolytic bacteria, such as *S. bovis*, produce lactic acid in their digestion

processes. Other bacteria within the rumen utilize this lactate to grow, including species such as

Megasphera elsdenii and the *Selenomonas ruminantium* group. Because of this, lactate using

bacteria also correspond to low fiber, high concentrate diets where amylolytic, lactate producing

bacteria will also thrive. *S. ruminantium* saw an increase in abundance between 0600 and 1200 h

as well as M. elsdenii between 0600 and 1500 h. Both of these peaks represent the increase in

concentrate intake at 0900 h, while amylolytic bacteria also exponentially increase in microbial

concentration during this time to produce lactic acid.

Microbial biohydrogenation is the process of converting unsaturated FA to more

saturated end products as previously discussed. Microbial populations involved in

biohydrogenation correspond to a higher fiber diet. Both *B. fibriosolvens/Pseudobutyrivibrio* and

Butytivibrio hungatei quickly increased between 0600 and 1200 h with HL treatment. *B.

hungatei* slightly rose during this time with LH treatment, but *B. fibriosolvens/

Pseudobutyrivibrio* did not increase with LH treatment. Surprisingly, no significant increase in

microbial concentration was seen in either species around H feeding of LH treatment at 1100 h.

Ruminal anaerobic fungi are only approximately 5-10% of the total rumen microbial population, but are important in hydrolyzing ester linkages in cellulose and hemicellulose. Higher numbers of fungi have been found in the rumen of cows fed high fiber, poorly digestible diets. In relation to total bacteria, fungi population dramatically increased between 0600 and 1200 h during HL treatment, however, relative abundance of fungi did not increase until 0900 and 1500 h during HL treatment. Relative abundance of fungi also dropped between 1500 to 2100 h before increasing again after H fed of LH diet at 2200 h.

Ciliate protozoa mainly derive their nutrients by digesting feed carbohydrates, especially starch and sugars, so they predominate in low fiber, high concentrate diets. In comparison to total bacteria, both HL and LH treatments produced much lower microbial concentrations compared to the control diet. However, relative abundance of ciliate protozoa also followed this pattern. In general, fungi concentration peaked between 0900 and 1200 h in the HL diet while LH peaked near 0600 h.

Total bacteria concentrations varied greatly between different treatments. Overall, LH treatment produced an increase in total bacteria around 0700 h before feeding of L diet, while dropping off again before a slight increase after 1100 h feeding of H diet. In contrast to this, HL diet maintained a fairly constant abundance of total bacteria from 300 to 1500 h, with a sharp drop before 2100 h feeding of L diet and continuous rise again throughout the night.

Chapter 6

CONCLUSION

The objective of investigating the fatty acid composition of rumen digesta and the relative abundance of selected microbial populations in the rumen over the day was accomplished. The data supports the hypothesis that FA profile is affected by time of day, however, time of day is not a specific factor in relative changes in microbial populations for all microbial species quantified. Bacterial populations quantified were used to represent niches within the rumen, however lack of variation in change of microbial population over the day may be attributed to changes in other, non-quantified bacterial species within the same niches. Further research projects can look at a wider variety of microbial populations within the rumen to test for further time of day interactions. Also, projects can also focus on different time of feedings throughout the day or manipulating different dietary effects rather than just forage to concentrate ratio.

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