EVALUATION OF MEDIUM-CHAIN FATTY ACID SUPPLEMENTATION EFFECTS ON DAIRY COW PERFORMANCE AND RUMEN FERMENTATION

by

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Abstract

Medium-chain fatty acids (MCFA) are fatty acids with chain lengths between 6-12 carbons and are primarily metabolized by the liver, however they may be absorbed and metabolized by intestinal mucosa. Medium-chain fatty acids provide the animal with available energy quicker as compared to other fatty acids. Additionally, MCFA have been shown to have strong antimicrobial properties due to the amphiphilic structure. The objective of this research was to evaluate effects of medium-chain fatty acid supplementation on productivity, plasma energy metabolite concentrations, apparent total tract nutrient digestibility, rumen fermentation, and rumen microbial profile of lactating dairy cows. Thirty (n = 8 primiparous, n = 22 multiparous) Holstein cows in mid-lactation (637 ± 68.5 kg of initial body weight, 98.5 ± 27.4 d in milk; mean ± standard deviation) were used in a crossover design with 28-d periods. The MCFA supplement, consisted of 25% MCFA (containing 32% C8:0, 21% C10:0, 47% C12:0 on DM basis) and 75% carrier ingredient, was fed at 0.25% of dietary DM, replacing dry ground corn in control (CON). Total inclusion of MCFA was 0.063% of dietary DM. No differences were observed in dry matter intake, apparent total tract nutrient digestibility and body weight change between MCFA and CON. However, there was a negative relationship between pretrial milk yield and animal response to MCFA treatment in body weight change; higher producing cows tended to increase body weight to a less extent when MCFA was supplemented. Milk and milk component yields did not differ between treatment groups. However, a negative relationship between pretrial milk yield and animal response to MCFA treatment in milk protein yield; higher producing cows decreased protein yield to a greater extent when MCFA was supplemented. The MCFA supplementation tended to have higher minimum rumen pH (5.66 vs. 5.54; P = 0.08), and decreased daily fluctuation range of rumen pH (1.17 vs. 1.40; P = 0.02) compared to CON. However, duration of

acidosis (pH < 5.8, min/d) did not differ between treatment groups and ruminal total volatile fatty acid concentration and its profile did not differ between treatment groups. For rumen microbiota, chao1 index of bacterial community tended to be lower (10.9 vs. 11.6; $P = 0.07$) whereas Shannon index did not differ in MCFA compared to CON, and both indices did not differ for archaeal and protozoan communities between treatment groups. The MCFA treatment increased relative abundance of an unidentified family of bacteria that belongs to the Mollicutes class (0.04 vs 0.01%; $P = 0.02$) and tended to increase relative abundance of an uncultured bacterium that belongs to the Bacteroidetes phylum (0.48 vs. 0.37%; $P = 0.06$). The relative abundance of *Methanobrevibacter gottschalkii* increased when supplemented with MCFA (5.14 vs. 4.92%; $P = 0.03$). These results suggest that supplementation of MCFA at 0.063% dietary DM may not affect overall animal performance or total tract nutrient digestibility, but stabilize rumen pH by affecting rumen microbiota.

Preface

The research project, which is part of this thesis, received research ethics approval from the University of Alberta Animal Care and Use Committee for Livestock, Project name "Mid-chain fatty acids", AUP 3474, 19 December 2019. Cattle were cared for according to the guidelines of the Canadian Council on Animal Care.

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List of Abbreviations

ADF	Acid detergent fiber
ADG	Average daily gain
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
BCS	Body condition score
BHB	β-hydroxybutyrate
BW	Body weight
C8:0	Caprylic acid
C10:0	Capric acid
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
CCK	Cholecystokinin
CH_4	Methane
CON	Control
CP	Crude protein
DIM	Days in milk

DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
ECM	Energy corrected milk
EE	Ether extract
FA	Fatty acid
FCM	Fat corrected milk
FFA	Free fatty acid
H_2	Hydrogen gas
LCFA	Long-chain fatty acid
MCFA	Medium-chain fatty acid
MCT	Medium-chain triglyceride
ME	Metabolizable energy
MP	Metabolizable protein
MUN	Milk urea nitrogen
NEB	Negative energy balance
NE_L	Net energy of lactation
NDF	Neutral detergent fiber
OM	Organic matter
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction

rRNA	Ribosomal ribonucleic acid
SARA	Sub-acute rumen acidosis
SCC	Somatic cell count
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acid
TCA	Tricarboxylic acid cycle
TMR	Total mix ration
UFA	Unsaturated fatty acid
VFA	Volatile fatty acid

Chapter 1. Literature Review

1.1 Feeding Management of Fat Supplements

1.1.1 Feeding Fat to Dairy Cattle

The addition of fat in the diet of dairy cattle are typically used to increase the energy density of the diets (NRC, 2001). Beyond increasing the energy density of diets, fat supplementation increases absorption of fat-soluble nutrients (NRC, 2001). It is suggested that total dietary fat should not exceed 7% of dietary dry matter (DM) due to reduction in dry matter intake (DMI), milk fat percentages and fiber digestibility when feeding large amounts of fat (NRC, 2001). However, the optimal dietary fat content is dependent on a combination of factors, including type of fat, stage of lactation, milk production level, diet ingredients, temperature, and other feeding management strategies (NRC, 2001).

Early lactation cows may benefit from fat supplementation when animals experience a negative energy balance. An increase in dietary energy may potentially increase the energy balance allowing for increased milk yield, metabolic health, and reproductive efficiency (Onetti and Grummer, 2004). However, it is suggested that total dietary fat in fresh cow diets remain under 6% to prevent a depression in DMI, nutrient digestion and compromised rumen fermentation which would consequently reduce overall energy intake (Chilliard, 1993; Onetti and Grummer, 2004). In a review of 41 studies on supplemental fat, on average 3.6% of supplemental fat on a DM basis was fed, decreased DMI in both early and mid-lactation cows and the magnitude of DMI depression tended to be more significant in mid-lactation cows (Onettie and Grummer, 2004). The decrease in DMI combined with an increase in milk production improves feed efficiency, indicating a potential financial benefit to producers.

Several studies have found significant interactions between fat supplements and feed ingredients. Smith et al. (1993) found a reduction in milk and milk fat yield when cottonseed and tallow was added to a corn silage diets. Depression of milk yield and milk fat can be overcome by replacing 25 or 50 percent of corn silage DM with alfalfa hay (Smith et al., 1993; Onettie et al., 2002). Rumen pH is increased when 50% of corn silage is replaced with alfalfa hay (Hassanat et al., 2013) and alfalfa silage (Onettie et al., 2002) suggesting increased biohydrogenation of fatty acids in the rumen (Martel et al., 2011).

Animal environment such as housing and temperature also play a major role in the responses to fat supplements. It has been suggested that fat supplementation is more suitable to increase energy intake by heat-stressed cows due to minimal rumen digestion of fat which decreases metabolic heat production and increases energy supply to the cow (West, 2003). Drackley et al. (2003) observed that increasing dietary fat from 2.6% to 6% in heat-stressed mid-lactation cows increased milk production and efficiency. Skaar et al. (1989) reported an increase in milk production during the warm season compared to the cool season when fat was used to increase dietary energy density. Using fat supplements to improve energy intake in animals experiencing heat-stress were found to increase milk production by 9% when animals were not housed with an evaporative cooling system (Chan et al., 1997).

As previously mentioned, the optimal dietary fat content is dependent on several factors (i.e., stage of lactation, milk production, diet ingredients, and temperature). These factors must be taken into consideration when selecting the best nutritional management strategies for optimizing animal performance.

1.1.2 Types of fat supplements

Fat sources have been divided into two categories, natural fats, and commercial fat products (Palmquist and Jenkins, 2017). Natural fats are oilseeds or animal fat whereas commercial fats are extruded from plant or animal source for a specialized feeding program (Palmquist and Jenkins, 2017). Fat supplements contain various fatty acid chain lengths but most commonly range from 16 to 18 carbons for their higher energy potential (NRC, 2001). Each fat type may produce various responses by the animal due to ruminal interactions, saturated vs. unsaturated and chain lengths (NRC, 2001).

1.1.2.1 Natural Fats

Oilseeds are the major source of plant fat used in diets of dairy cows, and containing high amounts of linoleic, linolenic, and oleic acids (Glasser et al., 2008). In North America, the most common oilseeds are cottonseed, soybeans, sunflower seed, and canola (USDA, 2021). Dietary inclusion of unprocessed oilseeds differs in their effects on animal performance. Whole cottonseeds are high in protein, fat, fiber, and energy, therefore, easily balanced in the ration of dairy cows (Arieli, 1998). Feeding whole cottonseeds increases milk and milk fat production (Anderson et al., 1979; Grainger et al., 2008). In addition, whole cottonseed decreased methane emissions whereas rapeseed and linseed increased methane emissions (Munoz et al., 2019).

Feeding whole roasted soybeans increased milk and milk protein yield (Knapp et al., 1991). Supplementation of sunflower seed, flax seed and canola seed increased dry matter intake (DMI) in lactating dairy cows when replacing calcium salts of long-chain fatty acids (LCFA), however milk yield was not affected, suggesting a decreased feed efficiency (Beauchemin et al., 2009). Up to 14% of dietary dry matter (DM), ground canola seed can be included in diets of lactating dairy cattle without having negative effects on performance (Chichlowski et al., 2005). Rapeseed oil can

be supplemented up to 7% of dietary DM to hay based diets without affecting rumen digestibility of nutrients (Ben Salem et al., 1993).

Tallow is a common animal fat fed to dairy cows and contains oleic acid in the highest abundance (Eastridge and Firkins, 2000; Ockerman and Basu, 2014). When supplementing tallow to an alfalfa silage-based diet milk fat and protein yields are increased (Ruppert et al., 2003). Supplementing tallow to diets with corn silage as the main forage, milk production is increased (Onetti et al., 2002). This suggest that the basal diet composition (i.e., alfalfa silage or corn silage) may interact with the fat supplement resulting in varying animal performance.

1.1.2.2 Commercial Fats

The majority of commercial fats are produced to bypass or have minimal effects on the rumen microbes to prevent negative effects of fat supplementation such as decreased fiber digestibility (NRC, 2001). Granular fats are designed to have minimal effects on rumen fermentation include calcium salts and hydrolyzed fat (NRC, 2001; Palmquist and Jenkins, 2017). Whereas protected fats are encapsulated so ruminal microbiota remain unaffected (NRC, 2001). Additionally, fatty acids (FA) may be obtained from various fat source or synthetic FA are produced to target feeding specific FA.

1.1.3.2.1 Calcium Salts

Calcium salts are rumen inert fat supplement made from palm oil, soybean oil or a blend of fat sources (Palmquist and Jenkins, 2017). Fatty acids are associated with a calcium ion instead of a glycerol backbone. At high temperatures and normal pH, calcium salts are a solid, thus having low solubility in the rumen (Chalupa et al., 1984; Schneider et al., 1988). It is important to note that calcium salts are efficient in preventing negative effects on rumen microbiota, fiber digestion and biohydrogenation of FA. (Jenkins, 1993; Van Nevel and Demeyer, 1996a). At normal rumen

pH, 60% to 90% of calcium salts pass through the rumen (Sukhija and Palmquist, 1990; Klusmeyer and Clark, 1991). In the abomasum, calcium ions are separated from free fatty acids (FFA) by the low acidity (Schneider et al., 1988). If dietary calcium is in excess or inadequately absorbed, calcium soaps may reform in the large intestine and prevent absorption of calcium (Jenkins and Palmqueist, 1984). Supplementing calcium salts have been shown to linearly decrease digestibility of calcium (Schneider et al., 1988; Schauff and Clark, 1992). Thus, when feeding large amounts of calcium salts, calcium reabsorption from bone may occur to meet maintenance and milk production requirements (Schauff and Clark, 1992).

In a 41-study review of fat supplementation, Onetti and Grummer (2004) found that supplementing calcium salts of palm oil between 2.2% to 6.1% of dietary DM increased milk yield by 1.29 kg per day while increasing total tract NDF digestibility by 2.1% of NDF. Calcium salts of palm oil increased total tract NDF digestibility compared to the control due to calcium salts having relatively low solubility in rumen fluid and having less interactions with rumen microbes (Onetti and Grummer, 2004). In addition, supplementing calcium salts of palm fat, NDF digestibility was 2.3 times higher than the digestibility of unprotected palm fat (Weiss and Wyatt, 2004). Fiber digestion is correlated to the adhesion of bacteria to feed particles, it has been shown that calcium improves this adhesion (Bayourthe et al., 1994). Ultimately, calcium salts of palm fat increase the digestible energy, increasing milk yield and feed efficiency (Weiss and Wyatt, 2004).

1.1.2.2.2 Hydrogenated Fats

Hydrogenation is the process of adding hydrogen ions to carbons with a double bond to reduce the number of unsaturated fatty acids (UFA) and increase saturated fatty acids (SFA). Unsaturated fatty acids can prevent growth of microbial populations and lead to milk fat depression (Jenkins et al., 2008). Hydrogenated fat supplements were developed to bypass

ruminal digestion and prevent negative consequences of feeding fat on rumen fermentation. Dry matter intake increases when unsaturated fatty acids are replaced with hydrogenated fatty acids (Harvatine and Allen, 2005). When feeding hydrogenated tallow, DMI was not affected, however milk yield and fat-corrected milk yield increased (Eastridge and Firkins, 1991). In addition, production performance did not differ when hydrogenated tallow was fed at 2% and 5% of dietary DM (Eastridge and Firkins, 1991). Fiber digestibility was not affected by inclusion of hydrogenated fat supplements (Eastrdige and Firkins, 1991; Harvatine and Allen, 2006a). It has been observed that supplementation of hydrogenated fat increases rumination time per day (Harvatine and Allen, 2006b; Oyebade et al., 2020). This increase in rumination time has been attributed to reduced ruminal motility and increase duodenal flow of saturated FA when supplementing hydrogenated fat (Harvatine and Allen, 2006b).

The degree of saturation may affect FA digestibility. Unsaturated fatty acids have a higher iodine value than compared to saturated fatty acids (Firkins and Eastridge, 1994). A higher iodine value of fat supplements is associated with a higher degree of FA digestibility (Firkins and Eastridge, 1994). Supplementation of hydrogenated tallow with 95% of FA saturated (Eastridge and Firkins, 1991) and hydrogenated yellow grease with 98% of FA saturated (Jenkins and Jenny, 1989) decreased total tract digestibility of fatty acids. Harvatine and Allen (2005) observed a decrease in plasma free FA concentrations when supplementing hydrogenated fatty acids with 80% of FA saturated.

1.1.3 New Research in LCFA supplementation

The addition of supplemental fat in the diet of dairy cows is commonly practiced to increase dietary energy density and support milk production. However, in recent years, the effects of specific LCFA supplementation on intake, nutrient digestion, metabolism, and

performance of dairy cows has received attention. The focus has been on the supplementing palmitic (C16:0), stearic (C18:0) and oleic acids (C18:1) (Lock and de Souza, 2018).

Feeding C18:0 may be the optimal FA to supplement to lactating cows because C18:0 is the main end product of biohydrogenation (Palmquist et al., 2005). Increasing dietary inclusion of C18:0 increases DMI (Piantoni et al., 2015; Boerman et al., 2017). Response in milk production and milk fat yield may vary by animal production level when supplementing C18:0. Piantoni et al. (2015) observed a greater increase in milk yield and milk fat yield in high-producing cows when supplementing C18:0, potentially suggesting a change in gene expression in the mammary gland. Boerman et al. (2017) observed no effect on milk fat yield when supplementing C18:0 at 2.30% of dietary DM, whereas Piantoni et al. (2015) observed an increase in milk fat yield when supplementing C18:0 at 1.92% of dietary DM. It is possible that digestibility of FA decreased when supplementing a higher amount of C18:0. However, Piantoni et al. (2015) observed a low transfer of absorbed C18:0 into milk and concluded that a large portion of supplemented C18:0 was oxidized by hepatic tissues, thus decreasing its export in milk which may explain why Boerman et al. (2017) observed no effect on milk fat yield.

A meta-analysis on C18:0 digestibility reported that the digestibility of C18:0 linearly decreases as its duodenal flow increases (Boerman et al., 2015). This linear decrease in digestibility may be due to high competition of lysolecithin or absorption sites (Boerman et al., 2017). Lysolecithin aids in FA solubilization, which is required for absorption (Freeman, 1969). In the previously mentioned meta-analysis, increasing duodenal flow of C16:0 did not affect the digestibility of C16:0 (Boerman et al., 2015). It may be due to lower melting point or smaller prill size of the fat supplement, which increases digestibility (Boerman et al., 2017; de Souza et al., 2017).

The greater digestibility of C16:0 may be more beneficial to increasing milk and milk fat yield of lactating dairy cows. Piantoni et al. (2013) observed no effect of C16:0 supplementation on DMI, however milk and milk fat yield were increased when compared to the control. Despite total fatty acid digestibility decreasing when supplementing C16:0, total FA absorbed increased (Piantoni et al., 2013). The increase in milk yield is likely due to the increase in energy consumed when supplementing C16:0. Increased plasma FFA concentrations observed when supplementing C16:0 (Piantoni et al., 2013), may be associated with increased milk fat yield (Kronfeld, 1965). However increased milk fat yield when supplementing C16:0 may be due to the observed increase in NDF digestibility and plasma CCK concentrations which are associated with longer retention time of digesta in the rumen (Piantoni et al., 2013). Increasing retention time of digesta in the rumen may decrease C18:2 trans-10, cis-12 FA, which is associated with milk fat depression, by complete biohydrogenation (Baumen et al., 2011).

Furthermore, the ratio of C16:0 to C18:0 of fat supplements on animal performance has been evaluated. Fat supplements with higher C16:0 increased milk energy output and milk fat yield whereas increasing C18:0 reduced nutrient digestibility causing lower performance compared to supplements high in C16:0 (de Souza et al., 2018; Western et al., 2020a). When comparing supplementation of C16:0 versus C18:0, Rico et al. (2014) observed no difference in DMI or milk yield. However, C16:0 increased feed efficiency, and yields of milk fat and fat corrected milk (Rico et al., 2014). Supplementing C16:0 increased plasma FFA concentrations (Piantoni et al 2013; Rico et al., 2014) which contributed to a greater supply of preformed FA thus resulting in greater milk fat yield (Rico et al., 2014).

Supplementation of C18:1 has been shown to increase nutrient digestibility, milk yield and shift the energy partitioning pathway towards BW (de Souza et al., 2019; Western et al.,

2020b). Prom and Lock (2021) suggested that C18:1 supplementation over long periods would increase body weight. De Souza et al. (2019) observed a linear increase in body weight change per day and blood plasma insulin concentrations when supplementing C18:1 in replacement of C16:0. It is suggested that C18:1 increases insulin secretions thus inducing lipogenesis in adipose tissue (de Souza et al., 2019).

The current knowledge of feeding LCFA to dairy cows suggest that feeding a combination of C16:0, C18:0 and C18:1 may optimize animal performance. Previous research has shown that decreasing the dietary ratio of C16:0 to C18:1, linearly increases milk yield (de Souza et al., 2019); whereas, decreasing the dietary ratio of C18:0 to C18:1, milk production is not affected, and feed efficiency is increased (Prom and Lock, 2021). Increasing C18:1 dietary inclusion linearly increases FA digestibility and preformed FA in milk when supplemented with C16:0 (de Souza et al., 2019) and C18:0 (Prom and Lock, 2021). Unsaturated FA have greater solubility, improving FA transfer to micelles (Freeman, 1969) and increased uptake and re-esterification in enterocytes compared to saturated FA (Ockner et al., 1972). However, there is a lack of research on dairy cattle production performance when supplementing a combination all three FA, C16:0, C18:0 and C18:1.

1.2 Ruminal Fermentation

In monogastric animals, anaerobic microbial digestion of carbohydrates and plant cell walls occurs at the end of the digestive tract, whereas ruminants have a larger fermentation complex located at the beginning of the digestive tract (Moss et al., 2000). This fermentation complex is referred to as the rumen and consist of two chambers: rumen and reticulum; allowing

for greater microbial efficiency to digest feedstuffs (Moss et al., 2000). Ingested feedstuffs are degraded in the rumen by endogenous and microbial enzymes (Moss et al., 2000).

Anerobic fermentation of carbohydrates and plant matter result in accumulations of volatile fatty acids (VFA), carbon dioxide, methane, and microbial proteins (Prins, 1979). It is estimated that VFA contribute approximately 70% of the energy requirements of cattle (Bergman, 1990). The synthesis of microbial proteins in the rumen account for more than 50% of amino acids entering portal circulation from the small intestine (Moss et al., 2000). Approximately 90% of total methane release originates from the rumen (Murray et al., 1976).

1.2.1 Microbiota

The rumen microbiota consists of bacteria, protozoa, archaea, and fungi. Bacteria have the largest population at approximately 10^{11} cells/g of rumen content, and digest carbohydrates and protein (Russell, 2002). Protozoa are the second most abundant in the rumen at approximately 10^{6} cells/ml of rumen fluid (Sato et al., 2010). Most protozoa obtain nutrients through phagocytosis of other microbes as well as digest carbohydrates and proteins (Russel, 2002). Archaea population counts present approximately 10^{8} cells/ml of rumen fluid (Matthew et al., 2019). Fungi populations are present at approximately 1.8×10^{4} zoospores/ml of rumen fluid (Khenjornsart and Wanapat, 2010). Fungi can hydrolyse ester links between lignin and hemicellulose or cellulose of plant material that is generally more difficult to digest (Srinivansan et al., 2001).

1.2.1.1 Bacteria

Rumen bacteria are localized within the rumen: suspended in rumen fluid, attached to feedstuffs, and adhered to the rumen wall (Sadet et al., 2007). Most commonly, bacteria are known for digestion of structural and non-structural plant carbohydrates and conversion into VFA. Bacteria possess the enzymes necessary to hydrolyze linkages between complex carbohydrates

(Sues, 2005). However, bacteria can also digest starch by utilizing amylase, producing VFA as an end product (Hobson et al., 1981). The *Prevotella* species is one of few species that can ferment amino acids for ATP production and bacterial growth (Rychlik et al., 2002). Bacteria are also able to synthesize all eight vitamin B compounds and vitamin K (Strobel, 1992; Nagaraja et al., 1997), providing the host animal with these vitamins after passing to the small intestine. Bacteria that are attached to the rumen wall are often involved in hydrolysis of urea entering the rumen (Fay et al., 1979), tissue recycling (McCowan et al., 1978), oxygen scavenging (Pacifico et al., 2001), and barrier functions such as biofilm formation (Macfarlane and Dillon, 2007).

Though the bacterial populations in the rumen are large, they are easily susceptible to change based on several factors including: diet and feeding regimen (Tajima et al., 2001; Rustomo et al., 2006), age of the animal, antibiotic usage, health of the host animal, genetics (e.g., Holstein v. Jersey; Kleen et al., 2003) and geographical location (Bryant, 1959). Tajima et al. (2001) observed a 20-fold increase in the quantity of fibrolytic bacteria three days after non-lactating cows switched from a hay-based diet to a grain-based diet. Rumen pH also affects bacterial growth. Cellulolytic bacteria cease growth when pH drops below 6.0 (Russell and Dombrowski, 1980). Brown et al. (2006) suggested that when pH is reduced, populations of bacteria are changed rather than diversity of bacteria.

1.2.1.2 Protozoa

It is estimated that protozoa may account for up to 50% of the ruminal biomass and can be classified in to two specific groups, entodinomorphs and holotrichs (Williams and Coleman, 1992; Williams et al., 2020). Protozoa have adapted to the rumen by finding niches (e.g., feed particles, epithelial wall) during ruminal contraction to avoid the flow into the intestines is largely due to a slow generation time ranging from 6 to 55 hours (Abe et al., 1981; Karnati et al., 2007). Protozoa

have obtained many of their functionally important genes from bacteria via horizontal gene transfer (Ricard et al., 2006). Approximately 75% of genes encoding for enzymatic processes and metabolism have been obtained from bacteria or as part of the symbiotic relationship (Williams et al., 2020).

Though protozoa account for approximately one-third of fiber digestion in the rumen (Russell and Rychlik, 2001), protozoa are not essential to animal survival (Williams and Coleman, 1992). Bacteria may be engulfed and degraded by protozoa, and used as the main source of protein for protozoa (Williams and Coleman, 1992; Belanche et al., 2012). The removal of protozoa from the rumen increases intestinal flow of microbial protein and the amino acid supply of leucine, threonine, and arginine (Newbold et al., 2015). Protozoa may be beneficial when feeding highly fermentable carbohydrates due to holotrichs protozoa ability to engulf and accumulate starch and soluble carbohydrates which when fermented by bacteria decreases pH and increases lactic acid acidosis (Williams and Coleman, 1992; Newbold et al., 2015).

1.2.1.3 Methanogens

Methanogens are group of archaea belonging to the phylum *Euryarcheota* (Hook et al., 2010) and grow at a neutral pH between 6-8 (Mathison et al., 1998). In lactating dairy cattle, *Methnobrevibacter ruminantium* is the largest group of methanogens (Whitford et al., 2001). Methanogens differ from bacteria in that they lack peptidoglycan in the cell wall and replaced by pseudomurein in *Methenobrevibacter* and *Methenobacterium*, heteropolysaccharide in *Methenosarcina* and protein in *Methanomicrobium* (Hook et al., 2010). Uniquely all methanogens have coenzyme F_{420} and coenzyme M (Hook et al., 2010). Coenzyme F_{420} is essential for hydrogenase and formate dehydrogenase enzymes whereas coenzyme M is methylated to produce methane (Hook et al., 2010).

For their important role in hydrogen removal, methanogens have symbiotic relationships with other organisms in the rumen. One example of this is hydrogen producing bacteria, *Ruminococcus albus*. When grown in the presence of a methanogen, partial pressure of H_2 is kept low through the formation of CH_4, allowing *R. albus* to continue producing H_2, and increase its energy efficiency to form acetate and ATP (Prins, 1979). Methanogens have formed and ecto- and endo-symbiosis relationship with protozoa and relies on interspecies hydrogen transfer (Finlay et al., 1994; Sharp et al., 1998). Hydrogen is inhibitory to protozoa metabolism and therefore benefit from removal of H_2 by methanogens (Sharp et al., 1998). Approximately 9% and 37% of methanogenesis can be attributed to the relationship between methanogens and protozoa (Finlay et a., 1994; Machmuller et al., 2003).

1.2.2 Identification of rumen microbiota using molecular techniques

1.2.2.1 Microbial Markers, PCR and Primer Selection

Microbial markers, such as coding and non-coding regions of genome, play an essential role in genetic research and rumen microbiota classification (Liu et al., 2012). Several characteristics are necessary of microbial markers: conserved gene must be present in all species; high polymorphic variation, and high conserved region of DNA (Yamamota and Harayama, 1996). For taxonomic identification, the 16S rRNA gene in bacteria and archaea as well as the 18S rRNA gene in protozoa are commonly used markers due to their uniqueness within individual species and resistance to mutation (Clarridge, 2004; Liu et al., 2012). However, some limitations exist when using 16S rRNA gene, closely related species that lack a high degree of polymorphic variation may be difficult to distinguish differences (Liu et al., 2012), and strains of the same species will not be identified.

Polymerase chain reaction (PCR) is one of the most widely used techniques used in the amplification of DNA segments (Hyeladi et al., 2019). Many rumen microbes cannot be grown by cultivation techniques for taxonomic identification and quantification (Kim et al., 2011; Hyeladi et al., 2019), thus PCR has provided a better understanding of microbes and their functions in the rumen. During PCR, millions of copies of target DNA are replicated in an exponential fashion over a series of cycles; denaturation, annealing and elongation (Ginzinger, 2002). However, PCR may be limited by the polymerase enzyme, primer selection, template dilutions, annealing temperatures, number of cycles, etc. (Wu et al., 2010). For example, a low number of cycles may cause higher estimation of taxa richness and a high number of cycles increases point mutation artifacts (Wu et al., 2010). In addition, the rate of PCR reaches a plateau phase, where copies of template DNA are not generating at an exponential rate causing quantification of PCR products unreliable (Ginzinger, 2002).

One of the most critical components of PCR is the selection of oligonucleotide primers. There are two main focuses when selecting primers for PCR: specificity and efficiency of amplification (Dieffenbach et al., 1993). Specificity is the frequency to which mis-priming events occur whereas efficiency is the primers ability to amplify target DNA at an exponential rate (Dieffenbach et al., 1993). Many factors influence the specificity and efficiency of primers such as the stability of the 3' end (Onodera and Melcher, 2004; Miura et al., 2005), melting temperature (Chavali et al., 2005), primer length (Dieffenbach et al., 1993), and GC content (Anderson et al., 2008). Primers with weak specificity may often produce more unrelated amplicons (Dieffenbach et al., 1993).

1.2.2.2 Amplicon Sequencing

As an efficient and cost-effective strategy, amplicon sequencing is a widely used tool for identifying taxonomic groups within microbial communities (Lundberg et al., 2013; Rauch et al., 2019). When using 16S rRNA gene sequencing, amplicon sequencing is generally limited to genus level identification (Walsh et al., 2018). Sequences are clustered into Operational Taxonomic Unit (OTU) based on similarities (Rideout et al., 2014). Typically, 97% similarity is sufficient to assign sequences to a species (Koeppel and Wu, 2013), however, some species have greater than 97% similarities in 16S rRNA sequences. One examples of this high similarity between species are *Clostridium botulinum* and *Clostridium sporogenes* (Rossi-Tamisier et al., 2015). Improving references databases and aligning sequences to pre-defined reference sequences may prevent these similarities from not reflecting the true microbial composition (Rideout et al., 2014). Additionally, amplicon sequencing is limited to identifying known sequences due to primer design (Ross et al., 2012).

1.2.2.3 Metagenomics

Metagenomics involves the analysis and taxonomic assignment of genomic DNA that has been isolated from an entire microbial community and can provide potential functional information (Walsh et al., 2018). This method has been vastly used to study interactions between human health and gut microbiome (Turnbaugh et al., 2006; Zhang et al., 2009). However, in the past twelve years, the use of metagenomic techniques to study cattle rumen microbiomes has taken off. Brulc et al. (2009) successfully identified four microbial genes encoding carbohydrate degrading enzymes. Khafipour et al. (2009), observed a decrease in the proportion of *Bacteroidetes* in cattle induced with sub-acute rumen acidosis. In addition, Li et al. (2019) overserved differences in rumen microbiota in cattle of opposite sex. Using metagenomic

techniques to study the rumen microbiome role with the host, future research may be able to improve management practices of cattle through diet, antibiotic use, and environmental factors.

1.2.3 Methanogenesis

Hydrogen gas is another major end product of anerobic fermentation of protozoa, fungi and some bacteria, however it does not accumulate in the rumen due to interspecies hydrogen transfer (Moss et al., 2000). Interspecies hydrogen transfer is the relationship between fermenting species and hydrogen utilizing archaea, often referred to as methanogens. The removal of H_2 is necessary as H_2 may cause reduction in organic matter digestion and prevent microbial growth (Mathison et al., 1998). The main pathway for H_2 removal by methanogens is through the formation of CH_4: $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$ (Moss et al., 2000).

Additionally, there is a distinct niche of methanogens, *Methanosarcina*, that utilizes methanol and methylamines to produce methane (Hungate et al., 1970). During the production of acetate, formate is produced and can also be used as a substrate for CH_4 (Hungate et al., 1970). Removing H_2 from the rumen, methanogens allow other organisms involved in fermentation to perform optimally. However, the production of CH_4 takes away energy from that could be used for production purposes (Reynolds et al., 2011). Approximately 2-12% of energy loss derives from methanogenesis (Johnson and Johnson, 1995). Gross energy, the total energy in feed, minus energy lost to feces, urine, and gas (primarily CH_4) is referred to a metabolizable energy, the dietary energy available for metabolism (Reynolds et al., 2011).

1.2.5 VFA Production

Volatile fatty acids are the primary energy source for ruminants which are produced by rumen microbes during fermentation. Depending on diet composition and time after feeding, total VFA concentrations in the rumen is highly variable, typically between 60 to 150 mM (Bergman,

1990). Acetate tends to be the most predominant VFA under most feeding conditions. Molar ratio of acetate to propionate to butyrate is generally 65:20:15 (Bergman, 1990). Microbial fermentation of 1 mol of hexose may produce 2 mol of acetate or propionate, or 1 mol of butyrate (Bannink et al., 2006). Diets high in starch may favor propionate production, thus reducing the molar ratio of acetate to propionate (Bergman et al., 1990). Only small amounts of glucose are absorbed in the intestinal tract (Bergman et al., 1970), propionate production is important as it contributes to net glucose synthesis (Bergman, 1990).

The extent of VFA production is affected by several factors including: the rate of carbohydrate digestion (Allen, 1997), digesta passage rate (Robinson et al., 1987), microbial populations and efficiency (Sniffen and Robinson, 1987), and interaction between feed and rumen microbes (Allen, 1997).

1.2.6 Ruminal pH

The rumen pH is typically held between 6 and 7 (Bryant, 1970), however, low rumen pH or acidosis is the most important concern when formulating diets as it may have impacts on animal health and performance. Rumen pH is determined by the balance between VFA production and removal of acid through absorption, neutralization and digesta passage (Allen, 1997). After the consumption of diets high in rapidly fermentable carbohydrates, VFA production increases, as a result, there is a decrease in rumen pH, upsetting the balance of acid production and removal. As rumen pH drops, growth and enzymatic activities of microbes decreases, resulting in less fiber digestion (Nocek, 1997; Beauchemin et al., 2003). Furthermore, at low rumen pH, the conversion of pyruvate changes from VFA to lactate, which is the strongest acid in the rumen, and may cause a server depression in DMI, laminitis, ulcerations, and liver abscesses (Allen, 1997; Owens et al., 1998).

Maintaining a narrow daily range of rumen pH may allow for more consistent VFA removal and energy supply (Oba and Allen, 2000). Volatile fatty acid absorption is partially regulated by rumen pH; however, a weak negative correlation was observed (Allen, 1997). At high pH, VFA are in the dissociated form and absorption rate is decreased (Ash et al., 1963), whereas, at a low pH, VFA are in the associated form and absorbed via simply diffusion (Allen, 1997).

1.3 Medium-chain Fatty Acid

Medium-chain fatty acid (MCFA) often found in coconut oil and milk, which contain approximately 66% and 11% MCFA, respectively (Hollmann and Beede, 2012; Vyas et al., 2012). Supplementation of MCFA in the diets of dairy cattle is not a widely adopted practice in industry, however MCFA supplementation has been studied in efforts to reduce methanogenesis, improve health, improve energy balance, and promote growth in ruminants and monogastric animals.

In ruminant research, MCFA have been supplemented in multiple forms including coconut oil, calcium salts of MCFA, and varying percentage of pure caprylic acid, carpric acid and lauric acid. Coconut oil is highest in lauric acid (C12:0) at 40% to 50%, followed by caprylic acid (C8:0) at 9% and capric acid (C10:0) at 6.5%, though there is a small amount of LCFA present (Hollmann et al., 2012; Boateng et al., 2016). The FA composition of calcium salts vary by manufactures; however, the FA profile of previous research has ranged between 20% to 25% of C8:0, 20% C10:0 and 55% to 60% C12:0 (Fukumori et al., 2013; Masadu et al., 2019).

In addition, MCFA exhibited a dose dependent response, when inclusion rate of MCFA increases in the diet, greater effects are observed. Supplementing coconut oil (Hollmann et al., 2012) and C12:0 (Faciola and Broderick, 2013) linearly decreased ruminal protozoa populations, methane production, NDF digestibility, and DMI as supplementation rates increased.

1.3.1 Comparison between MCFA and LCFA

1.3.1.1 Ruminal metabolism

Lipids entering the rumen are altered by microbes through two major pathways, lipolysis and biohydrogenation (Jenkins et al., 2008). In lipolysis, ester linkages are hydrolyzed by microbial lipases, separating the glycerol backbone from fatty acids (Dawson et al., 1977). After lipolysis, biohydrogenation occurs, which is the process of converting UFA to SFA (Jenkins et al., 2008). Isomerization of UFA produces trans FA, followed by hydrogenation of double bonds, transforming into SFA (Harfoot and Halewood, 1988). It is important to note that the rate of lipolysis and biohydrogenation is dependent on rumen pH (Van Nevel and Demeyer, 1996b) and type and amount of fat entering the rumen (Beam et al., 2000).

Eighteen carbon, polyunsaturated and monounsaturated FA are the most common in dairy cattle diets due to their high abundance in forages and grains. Shoreland et al. (1955) observed the conversion of linolenic acid (C18:3) to linoleic acid (C18:2) and C18:1 intermediate, and a final conversion to C18:0. Wood et al. (1963) observed that 48 hours after ruminal injection of C18:2, 46% of the dose had been completely saturated into C18:0, 33% to 50% of the dose had only been partially hydrogenated, while 3% to 6% was not biohydrogenated. The high rate of biohydrogenation to C18:0 causes milk fat FA composition to be high in C18:0 (Glasser et al., 2008). However, there is a small proportion of partially hydrogenated FA that escape the rumen, and they are incorporated into milk fat (Glasser et al., 2008). Under normal rumen condition, the proportion of partially hydrogenated FA do not pose a risk to milk fat synthesis, whereas at a low rumen pH, the efficiency of microbial hydrogenation decreases, leading to an increase in partially hydrogenated FA leaving the rumen. The high amount of trans fatty acids leads to milk fat depression (Bauman and Griinari, 2003).

1.3.1.2 Absorption

Fatty acid chain length can influence the route of absorption from the intestinal tract (Playoust and Isselbacher, 1964). Absorption of MCFA in the intestine is faster and more efficient than long-chain fatty acids due to MCFA not needing to bind to a transporter (Marten et al., 2006). The rapid absorption of MCFA has been observed in the upper gastrointestinal tract of pigs (Odle et al., 1991). When MCFA are immersed in pancreatic lipase or bile salt, absorption can still occur unlike LCFA (Bach and Babayan, 1982). Similar to short-chain fatty acids, MCFA is absorbed into the intestinal epithelium by simple diffusion (Schonfeld and Wojtczak, 2016).

Once absorbed into the epithelial cells, MCFA are directly metabolized by intestinal epithelium or transported to the liver as FFA via portal circulation (Hashim et al., 1964; Hanczkowska, 2017). The majority of MCFA are bound to albumin in blood for transportation (Spector, 1975). However, a limited proportion of MCFA may be incorporated into chylomicrons as triglycerides, reaching the systemic circulation via lymph system (Bach and Babayan, 1982).

In contrast, due to the low solubility in water, LCFA are incorporated into mixed micelles in the small intestine (Wang et al., 2013). Micelles act to facilitate passage of LCFA across the membrane of enterocytes (Wang et al., 2013). Long-chain fatty acids may be transported by diffusion (Hamilton et al., 2001) or a protein mediated mechanism (Poirier et al., 1996). Inside the intestinal enterocyte, LCFA are esterified to triglycerides and incorporated into chylomicrons (Schonfeld and Wojczak, 2016). Chylomicrons are then released into the lymphatic system before entering the blood circulation via thoracic duct (Wang et al., 2013). The secretion of lipoprotein lipase hydrolyzed chylomicrons to release FFA and glycerol (Wang et al., 2013).

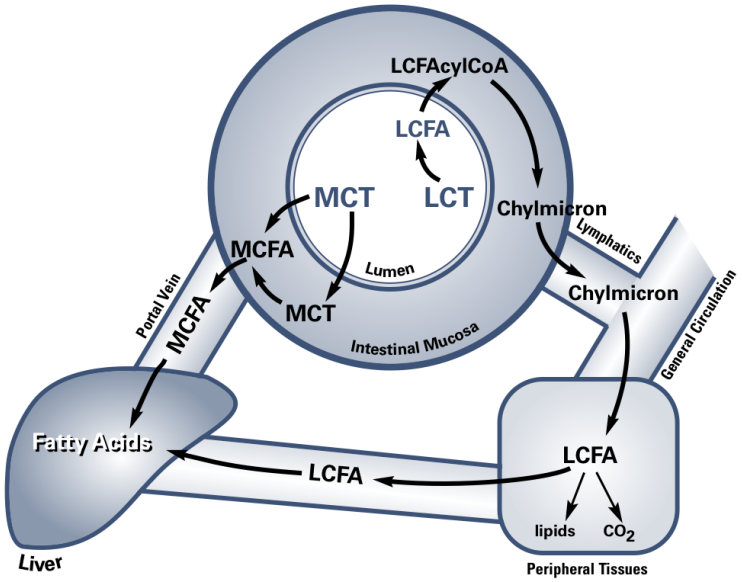

Figure 1.1 Intestinal absorption and transport of MCFA and LCFA from the lumen to liver (adapted from Dean and English, 2013)

1.3.1.3 MCFA Metabolism

In the liver or intestinal mucosa, MCFA are highly oxidative and metabolized as quickly as glucose (Babayan, 1987). Medium-chain fatty acids are efficiently used as an energy source though mitochondrial β-oxidation (Wojtczak and Schonfeld, 1993; Marten et al., 2006). In addition, MCFA can easily cross the mitochondrial membrane as they do not require carnitine palmitoyl transferase for intramitochondrial transport which is a regulating factor for LCFA oxidation (Marten et al., 2006). Once in the mitochondria, MCFA are activated by medium-chain acyl CoA synthase and are rapidly oxidized (Ikede et al., 1985; Marten et al., 2006). To oxidize MCFA, sufficient oxaloacetate is necessary to increase the entry of acetyl CoA into the TCA cycle (Marten et al., 2006). An excessive amount of acetyl CoA may increase ketone body production (Seaton et al., 1986). It is important to note the MCFA do not require fatty acid-binding proteins for intracellular metabolism, reducing the number of regulatory pathways (Marten et al., 2006; Ishizaea et al., 2015).

A small fraction of MCFA, which are not utilized by the liver, are distributed to peripheral tissue via blood circulation (Bach and Babayan, 1982). In addition, MCFA can be deposited into adipose tissue, which has been observed in rodents (Han et al., 2003), however MCFA can be useful in weight management. During a two-month feeding of medium-chain triglycerides reduced fat mass in rats through down-regulation of adipogenic genes (Han et al., 2003).

Diets high in long-chain saturated fats have been linked to insulin resistance (Riccardi et al., 2004) whereas C8:0 supplementation stimulate glucose-mediated insulin secretions (Stein et al., 1997). Oxidation of long-chain fatty acids tend to be depressed when diets are high in carbohydrates (Marten et al., 2006). Sidossis et al. (1996) found that an increased glucose supply decreases C18:1 oxidation but not C8:0. This suggests that concentration of glucose supply can control entry of LCFAs but not MCFA into the mitochondria.

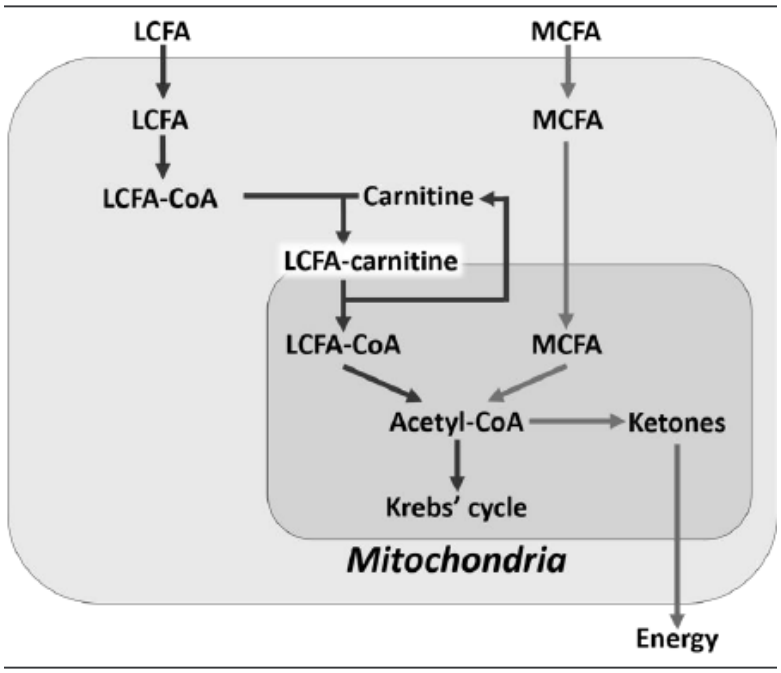

Figure 1.2 Mitochondrial metabolism of MCFA and LCFA in the liver or intestinal epithelium. (adapted from Nugent et al., 2015)

1.3.2 Antimicrobial effects of MCFA

Medium-chain fatty acids have been used in the preservation of silages (Woolford, 1975) due to the ability to inactivate pathogenic bacteria, viruses, and parasites (Zentek et al., 2011). Medium-chain fatty acids cause the membrane of bacteria to become unstable by the incorporation into the cell wall and cytoplasmic membrane along with the inhibition of bacterial lipases leading to lysis (Isaacs et al., 1995; Kim and Rhee, 2013). Another antimicrobial pathway of MCFA may be the potential cytotoxic effects from bacterial absorption of undissociated MCFA (Zentek et al., 2011). Medium-chain fatty acids can dissociate into protons and anions in the cytoplasm of the bacterial cell, decreasing pH (Zentek et al., 2011). This leads to inhibition of cytoplasmic enzymes and cell death (Freese et al., 1973).

In monogastric animals, the antibacterial effects of MCFA are assumed to be limited to the gastric stomach and the proximal small intestine (Zentek, 2011); however, the antibacterial effects begin in the rumen of ruminants. Due to the fact MCFA are rapidly absorbed and mostly found in the dissociated form at neutral pH and undissociated at pH between 3 and 6, acidification of the gastric stomach or rumen, may increase the antibacterial effects of MCFA (Dierick et al., 2002; Zentek et al., 2011). In vitro studies have shown an increase in antibacterial effects of MCFA to be greatest in the undissociated forms (Zentek et al., 2011). This may be advantageous for young monogastric animals or pre-ruminant calves, especially during the time of compromised immune systems such as the weaning transition period.

Dierick et al. (2002) reported a reduction in total *Escherichia coli* in the digesta of the stomach and duodenum of weaned piglets fed MCFA. Rabbits supplemented with C8:0 showed lower number of *E. coli* colonization in the feces after challenged with *E. coli* (Shrivanova et. al., 2009). Mice that were orally injected with C10:0 and *Vibrio cholerae* were found to have no traces

of the bacteria in the ileum or caecum suggesting that MCFA protect against intestinal infections (Petschow et al., 1998). One study supplementing C8:0 suggested *Campylobacter* can be controlled in broilers starting at 1 day of age (Solis de Los Santos et al., 2008).

Zinc oxide has historically been used a growth promoter with beneficial effects on the intestinal microflora to decrease the prevalence of diarrhea in piglets (Li et al., 2010). Since zinc is a heavy metal and toxic to some organs, Kuang et al. (2015) replaced zinc oxide with MCFA and observed weaned pigs consumed more feed and had higher daily gain. In the same feeding trial, pigs supplemented with MCFA had higher ileal and rectal counts of *Lactobacillus* (Kuang et al., 2015), which is beneficial in preventing diarrhea (Timmerman et al., 2005).

1.3.3 Monogastric Research

1.3.3.1 Intake

Pure MCFA often have an unpleasant smell, therefore medium-chain triglycerides (MCT) are often used in monogastric feeding (Hanczakowska, 2017). A linear decrease in feed intake was observed when piglets were supplemented with 0.7%, 1.4% and 2.1% of MCT in the diet (Li et al., 2015). When supplementing MCT at 12% of the diet, weaned piglets consumed 26% less feed (Price et al., 2013). Furuse et al. (1993) observed a decrease in feed intake when supplementing C8:0 and C10:0 at 20% of the diet to 23 d old chicks. Cave et al. (1982) observed a decrease in feed intake when supplementing C8:0, C10:0 and C12:0 at 0.3% of the diet to 21 d old chicks. One possible explanation for reduced feed intake is higher concentrations of blood free fatty acids following feeding of MCT in piglets or the faster oxidation of MCFA in the liver may induce satiety thus reducing feed intake (St-Onge and Jones, 2002; Hanczkowska, 2017).

1.3.3.2 Growth

When supplementing MCT, Li et al. (2015) observed a linear increase in average daily gain during the first 14 days after weaning, however, after 28 days of supplementing MCT, average daily gain did not differ. Supplementation of C8:0 and C10:0 had piglets maintained a higher average daily gain through 84 days of life (Hanczakowska et al., 2013). Studies that have shown increased weight gain in piglets when supplementing MCFA (Hanczakowska et al., 2011) could be attributed to the increase in protein digestibility and rapid oxidation of MCFA (Hanczakowska et al., 2017).

Hanczakowska et al. (2013) observed that piglets supplemented with 0.2% C8:0 improved apparent total tract digestibility of crude protein, crude fat, and fiber. Hanczakowska et al. (2011) supplemented piglets with C8:0, C10:0 and a combination of both FA, and observed greater intestinal villus height and crypt depth. Chwen et al. (2013) reported increased villus height in pre-weaned piglets supplemented with MCT along with increased body weight gain. The increase in body weight gain observed (Hanczakowska et al., 2011; Chwen et al., 2013) can be attributed to the increased surface area of the small intestine which is the primary site for nutrient digestion and absorption (Cera et al., 1988). Villus height is directly correlated to body weight gain in early weaned pigs (Zijlstra et al., 1996). Additionally, MCFA have been used as hydrophilic drug absorption enhancers, acting on tight junctions to regulate paracellular permeability (Lindmark et al., 1997). In pigs supplemented with C10:0, expression of genes regulating tight junctions increased, and restored impaired epithelial barrier functions after treatment with cyclophosphamide (Lee and Kang, 2017).

Shokrollahi et al. (2014) found an increase in breast yield of broilers supplemented with 0.1%, 0.2% and 0.3% of MCFA. Furthermore, Kuang et al. (2015) found an increase in dietary

amino acid digestibility in weaned pigs accompanied with an upregulation of CAT2 gene, an amino acid transporter, which could explain the increased average daily gain when supplementing MCFA. Excess amino acids can be converted to glucose which is subsequently oxidized to energy that can be used for protein synthesis (Shokrollahi et al., 2014). In addition, fat deposition in broilers is decreased when feeding MCFA (Chiang et al., 1990; Shokrollahi et al., 2014). Baltic et al. (2017) reported that MCFA are poorly deposited into subcutaneous fat in pigs and poultry, resulting in higher carcass quality.

In monogastric animals, MCFA have been shown to increase growth via increasing intestinal tract surface area and nutrient digestive capacity. As the intestinal tract of monogastric and ruminant animals are similar, these results suggest the dairy cattle may benefit from MCFA supplementation by increasing nutrient digestion. In addition, the decrease in fat deposition observed in pigs and poultry may be replicated in cattle thus preventing obesity and decreasing the risk of metabolic disorders.

1.3.4 Ruminant Research

1.3.4.1 Rumen Fermentation

Largely due to the antimicrobial activity of MCFA, rumen microbiota populations have significantly decreased when supplementing coconut oil or pure MCFA between 1% - 5% of dietary DM. When supplementing coconut oil in vitro, bacterial populations are reduced (Dohme et al,. 1999; Machmuller et al., 2001), however when supplementing coconut oil in vivo total bacterial populations remained unaffected (Patra and Yu, 2013). Bacterial populations when supplementing C12:0 were not affected (Dohme et al., 2001; Machmuller et al., 2001).

Supplementation of coconut oil in vitro, protozoal populations are almost eliminated from rumen fluid (Dohme et al., 1999; Machmuller et al., 2001), however in vivo protozoal populations

are decreased but still present (Hristov et al., 2009; Patra and Yu, 2013). *In vitro* supplementation of C12:0, protozoal populations were reduced (Dohme et al., 2001; Machmuller et al., 2001). Supplementing C12:0 *in vivo*, protozoal populations are reduced (Hristov et al., 2009; Hristov et al., 2011). In addition, a linear decrease in protozoal populations were observed when inclusion of C12:0 increased in the diet (Faciola and Broderick, 2013). In addition, supplementing coconut oil and C12:0 *in vitro* (Dohme et al., 1999; Dohme et al., 2001), as well as supplementing coconut oil *in vivo* (Patra and Yu, 2013), archaeal population are reduced.

The reduction in protozoal and archaeal populations observed when supplementing MCFA is often associated with a reduction in methane production. When supplementing coconut oil *in vitro*, methane production was decreased (Machmuller et al., 2001) and a linear reduction of methane production was observed *in vivo* (Hollmann et al., 2012). *In vitro*, methane production was increased when supplementing C8:0 and C10:0, whereas C12:0 decreased methane production (Dohme et al., 2001). However, *in vivo* supplementation of C12:0 had no effect on methane production, whereas coconut oil decreased methane production (Hristov et al., 2009). One possible explanation for coconut oil decreasing methane production is myristic acid, C14:0, which accounts for 18% to 20% of total FA in coconut oil (Hristov et al., 2009). Soliva et al. (2004) observed that a 4:1 ratio of C12:0 to C14:0 was more effective at reducing methane production as compared to only supplementing C12:0.

Beyond protozoal and archaeal population effects on methanogenesis, the differences in VFA profile, when supplementing MCFA, is related to methanogenesis as fermentation to acetate and butyrate promotes CH_4 production while propionate acts as a competitive pathway for hydrogen consumption (Moss et al., 2000). *In vitro*, Dohme et al. (2001) observed decreased molar proportion of propionate and increased methane formation when C8:0 and C10:0 were

supplemented, whereas C12:0 increased molar proportions of propionate and decreased methane formation when compared to C8:0 and C10:0. Machmuller et al. (2001) observed a decrease in methane emissions and increase in molar proportion of propionate when supplementing coconut oil *in vitro*.

When supplementing MCFA, many studies have observed decreased total tract NDF digestibility, likely due to reduction in rumen bacterial and protozoal populations (Hollmann et al., 2012; Reveneau et al., 2012; Faciola and Broderick, 2013). *In vitro*, NDF digestibility is decreased by C12:0 (Dohme et al., 2001; Machmuller et al., 2001) and coconut oil supplementation (Dohme et al., 1999; Machmuller et al., 2001). Lauric acid (C12:0) supplementation *in vitro* did not affect crude protein digestibility (Machmuller et al., 2001), whereas *in vivo* supplementation crude protein digestibility decreased (Faciola and Broderick, 2013). Coconut oil supplementation decreased crude protein digestion *in vitro* (Machmuller et al., 2001) and *in vivo* (Hollmann et al., 2012).

Due to the major reduction in fiber digestion by microbes when supplementing MCFA, it is expected that VFA production is reduced, and its profile is altered. Castro-Montoya et al. (2012) reported that MCFA significantly reduced total VFA production *in vitro*. Dohme et al. (1999) and Machmuller et al. (2001) reported significant decreases in total VFA concentrations *in vitro* when supplementing coconut oil. Based on previous research, the source and chain length of MCFA may affect VFA molar proportions. Dohme et al. (2001) reported a reduction in propionate concentration and increased butyrate when supplementing C8:0 and C10:0, whereas C12:0 increased propionate concentrations but had no effect on butyrate concentration. *In vivo*, Hristov et al. (2009) reported a decrease in propionate concentrations when supplementing C12:0, whereas

propionate was increased when supplementing coconut oil (Dohme et al., 1999; Hristov et al., 2009 and Machmuller et al., 2001).

When supplementing MCFA, the decrease in VFA concentrations (Dohme et al., 1999, Machmuller et al., 2001) would suggest rumen pH would be increased. However, *in vitro* rumen fluid pH was decreased when coconut oil was supplemented (Dohme et al., 1999; Machmuller et al., 2001), whereas *in vivo* rumen pH was not affected by C12:0 or coconut oil supplementation (Hristov et al., 2004; Reveneau et al., 2012; Faciola and Broderick, 2013). *In vitro* rumen fluid pH may have been decreased due to the observed decrease in ammonia concentrations (Dohme et al., 1999; Machmuller et al., 2001). Ammonia acts a buffer, accepting hydrogen ions in the formation of ammonium (Allen, 1997).

1.3.4.2 DMI

When supplementing MCFA to dairy cattle, many studies have reported decreased DMI, which was accompanied with decreased NDF digestibility (Hollmann et al., 2012; Reveneau et al., 2012; Faciola and Broderick, 2013). This suggest that DMI is limited by rumen fill and particle passage rate. Van Soest (1965) observed that DMI was more highly correlated with NDF than other chemical measurements. The reticulorumen is thought to be the limiting site for DMI when feeding high fiber diets (Baile and Forbes, 1974). As the fiber mass in the rumen increases, DMI is decreased (Allen, 1996). In addition, ruminal digesta mass was not affected when supplementing coconut oil (Reveneau et al., 2012), suggesting particle passage rate must be decreased to observe a decrease in DMI (Allen, 1996). Dry matter intake and particle retention time in the reticulorumen are negatively correlated (Allen, 1996). Reveneau et al. (2012) observed a decrease in particle passage rate when supplementing coconut oil. A decrease in NDF digestibility increases rumen fill and decreases particle passage rate, thus triggering satiety (Allen, 2000).

Hollmann and Beede (2012) reported a significant reduction in DMI after one day of supplementing coconut oil. Fukumori et al. (2013) reported a decrease in DMI when supplementing MCFA calcium salts. Potentially, rumen fill, and particle passage rate may not be the only reason for observing decreased DMI when supplementing MCFA, and instead MCFA may induce satiety by hepatic oxidation (Allen et al., 2009). Medium-chain fatty acids have been shown to elicit greater reductions in DMI compared to LCFA (Dohme et al., 2004; Hollmann and Beede, 2008) possibly due to faster absorption and transport to the mitochondria (Langhans, 1996).

In addition, palatability may also affect DMI. Grummer and Socha (1989) attributed the reduction in DMI to palatability when supplementing medium-chain triglycerides. With the addition of a flavoring agent or reducing the inclusion of MCFA in the diet, palatability issues may be avoided. The current body of knowledge suggest that MCFA decreased DMI most likely via rumen fill or hepatic oxidation.

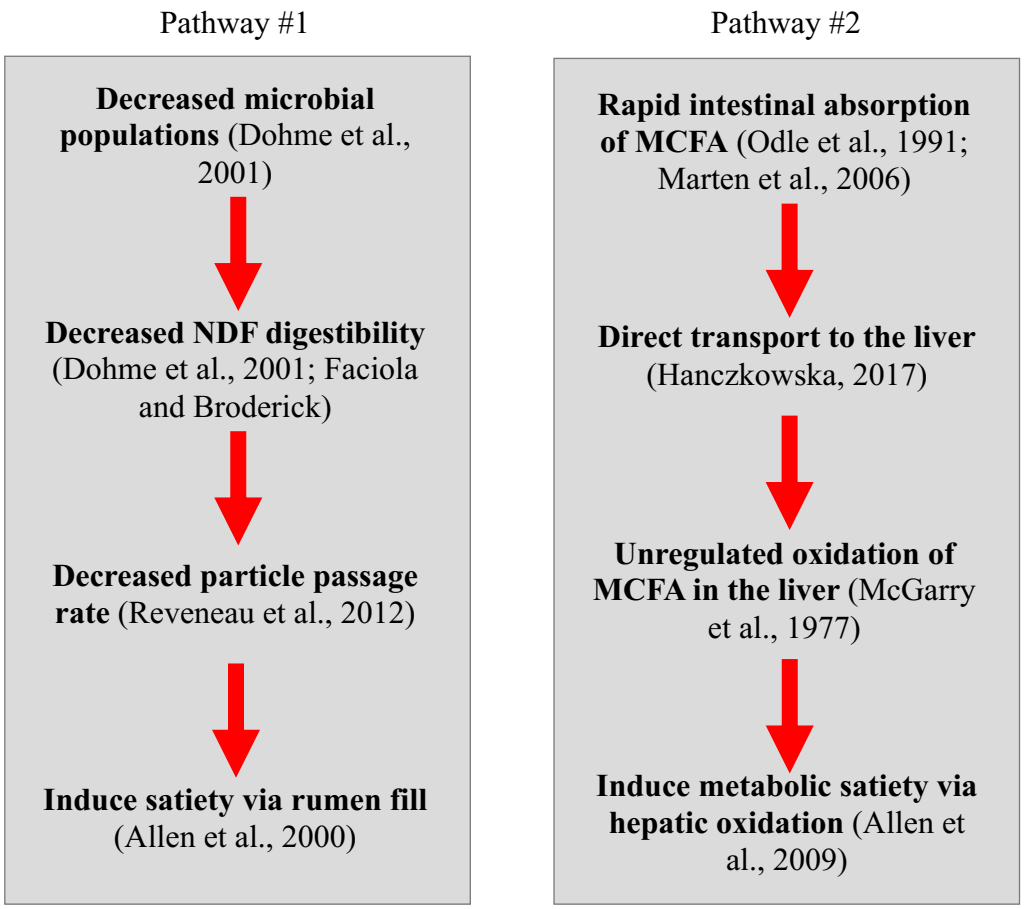

Figure 1.3 Pathway of reduced DMI when MCFA are supplemented.

1.3.4.3 Milk and Milk Component Yield

When MCFA are supplemented to dairy cattle, inclusion rates of MCFA in the diet influence milk yield and component yield by affecting DMI. Reveneau et al. (2012) observed that supplementing coconut oil at 5% of dietary DM reduced DMI and milk yield. Hollmann et al. (2012) observed a reduction in milk yield and DMI when supplementing coconut oil at 3.3.% of dietary DM, whereas coconut oil supplemented at 1.3% of dietary DM did not affect milk yield or DMI. Medium-chain fatty acid calcium salts supplemented at 1.5% of dietary DM tended to decrease milk yield due to a reduction in DMI (Fukumori et al., 2013). Faciola and Broderick (2013) observed a linear reduction in milk yield and DMI when C12:0 was supplemented at 0.97%, 1.95% and 2.92% of dietary DM. Intraruminal infusion of 240 g/d of sodium laurate did not affect

milk yield or DMI in late lactation cows (Hristov et al., 2004) whereas intraruminal infusion of 240 g/d of C12:0 reduced DMI leading to a reduction in milk yield in mid-lactation cows (Hristov et al., 2011).

Though milk yield often decreases when supplementing MCFA, supplementation of C12:0 linearly increased feed efficiency (Faciola and Broderick, 2013). Increasing feed efficiency may be very beneficial as feed cost accounts for up to 60% of total production cost (Connor, 2014), therefore, if the producer can save money on feed cost in the lactating herd, the success of the farm improves. Though this is dependent upon the cost of the MCFA supplement.

There are several factors that may affect milk component yield. Generally, if DMI is reduced, milk yield will be reduced, resulting in reduced milk component yield as well. In many studies supplementing MCFA, milk component yields have been reduced alongside DMI and milk yield (Reveneau et al., 2012; Faciola and Broderick, 2013). In late lactating cows, milk fat and protein yield were not affected by C12:0 supplementation (Hristov et al., 2004) whereas in mid-lactation cows milk fat and protein yield was reduced (Hristov et al., 2011). Supplementing coconut oil at 1.3% of dietary DM did not affect milk fat and protein yield, however 3.3% of coconut oil reduced fat and protein yield (Hollmann et al., 2012).

Milk fat depression (MFD) may be caused by several factors including reduction of DMI, decrease in apparent digestibility of nutrients, a shift in biohydrogenation (Hollmann et al., 2012; Faciola and Broderick, 2013) or in the case of MCFA supplementation, all three may contribute. Specifically, coconut oil has been shown to increase concentrations of C18:2 trans-10, cis-12 in milk fat (Hollmann et al., 2012), which is associated with MFD (Bauman and Griinari, 2003). Generally, this shift in milk fatty acid concentration is attributed to changes in ruminal biohydrogenation (Hollmann et al., 2012). Diets that provide high ruminally available starch and

low NDF, reduced ruminal pH can decrease the complete biohydrogenation of UFA (Sandri et al., 2020). Supplementing MCFA have been previously shown to decrease NDF digestibility, suggesting that as the ratio of ruminally digested NDF to starch decreases, the extent of biohydrogenation is decreased (Hollmann et al., 2012; Rico and Harvatine, 2013).

In previous research, MCFA have decreased milk lactose yield (Dohme et al., 2004; Fukumori et al., 2013; Bayat et al., 2018). Fukumori et al. (2013) attributed the decrease in lactose yield in multiparous cows to the increase in plasma β-hydroxybutyrate (BHB), which inhibits lactate removal and the production of glucose in the liver (Kummel, 1984). These findings suggest that inclusion of MCFA at > 1% of dietary DM may further decrease milk yield by reducing gluconeogenesis.

1.3.4.4 Blood Metabolites and Hormones

When supplementing MCFA to lactating dairy cattle, only a very few studies have assessed the effects on blood metabolites and hormones. Fukumori et al. (2013) observed a reduction in plasma concentration of glucose. Since the stimulus for insulin secretion from the pancreases is an elevation in blood glucose, it was also observed that insulin concentrations were decreased when supplementing MCFA (Fukumori et al., 2013). However, when supplementing MCFA to calves, post weaning glucose concentrations did not differ whereas insulin concentrations were reduced (Masadu et al., 2019). In calves, it is likely glucose concentrations did not differ due to MCFA exerting little effects on DMI and fermentation. Ruminal VFA production often reflects plasma glucose concentration (Wang et al., 2012). When feeding MCFA to lactating cows, the observed decrease in DMI led to a reduction in VFA production (Faciola and Broderick, 2013), decreasing glucose availability for milk production (Fukumori et al. 2012).

A decrease in insulin concentrations in lactating dairy cattle when supplementing MCFA may decrease body weight gain. Adipose tissue and muscle require insulin for glucose uptake (Zhou et al., 1999), suggesting that by decreasing insulin concentrations, adipose tissue growth from the uptake of glucose may be decreased. However, body weight in dairy cattle was not affected by MCFA (Hristov et al., 2009; Hristov et al., 2011).

In addition to decreased concentration of glucose in lactating cows when fed MCFA, concentrations of FFA have been observed to increase (Fukumori et al., 2012). Mobilization of FFA from adipose tissue is an indication of a negative energy balance (NEB); when energy requirements are higher than energy intake (Adequyi et al., 2005). This increase in FFA is likely due to the dramatic decrease in DMI experienced by animals when supplemented with MCFA. Another indication of NEB is increased concentration of BHB (Bell, 1995). Fukumori et al. (2012) observed an increase in BHB when supplementing MCFA. Acetyl CoA is formed from the oxidation of MCFA; oxaloacetate is necessary for acetyl CoA to enter the TCA cycle (Seaton et al., 1986; Marten et al., 2006). The rapid oxidation of MCFA may result in excess acetyl CoA (Marten et al., 2006), thus ketones are formed from two acetyl CoA molecules (Seaton et al., 1986). However, supplementing MCFA to weaned calves did not affect FFA or ketone concentrations in the blood possibly because DMI was not affected (Masadu et al., 2019).

1.3.4.5 Milk Replacer

Milk replacers are typically formulated using animal fat that are relatively low in MCFA as the main fat source (Esselburn et al., 2013). Calves experienced a higher average daily gain (ADG), pre and postweaning when milk replacer was supplemented with a blend of butyrate, MCFA and 18-carbon FA (Hill et al., 2011; Essleburn et al., 2013). It is likely that butyrate had the largest influence on ADG in calves as secretions of digestive enzymes have been reported to

increase thus potentially increasing nutrient digestibility (Guillotueau et al., 2009) and the contribution to rumen development (Sander et al., 1959), however Hiltz and Laarman (2019) did not observe a difference in ADG when supplementing butyrate in milk replacer. When supplementing a blend of FA, milk replacer and starter intake did not differ (Essleburn et al., 2013). Mills et al. (2010) supplemented milk replacer with caprylate oil (32% C8:0) and coconut oil (32% C12:0) and observed no effect on DMI or final body weight. However, supplementation of C8:0 decreased ADG whereas C12:0 did not affect ADG (Mills et al., 2010). This difference in ADG was attributed to cold stress experienced by the calves which led to inefficient use of energy as the calories from fat increased (Mill et al., 2010).

Calves fed a blend of butyrate, MCFA and 18-carbon FA showed decreased incidences of scours and the treatment for natural clostridium was reduced (Hill et al., 2011). Though not one source of FA can be verified as the source for this health improvement, it is likely that MCFA played an important role. The antimicrobial effects of MCFA may influence the establishment of intestinal microbiota. In pigs, *Lactobacillus* populations increased when supplementing MCFA (Kuang et al., 2015), which may be beneficial in preventing diarrhea in calves (Timmerman et al., 2005).

1.4 Knowledge Gap

Based on literature reviewed, two knowledge gaps have been identified when supplementing MCFA to lactating dairy cows.

1. Animal responses at a low inclusion rate of MCFA in the diet

Many studies have used MCFA supplementation as a method to mitigate methane production, therefore, previous research has supplemented MCFA at greater than 1% of dietary

DM, and methane emissions are successfully reduced. However, the antimicrobial properties of MCFA, reduces bacterial and protozoal populations leading to a decrease in ruminal NDF digestion. As previously stated, decreasing NDF digestibility reduces DMI and limits the nutrients available for milk production causing milk and milk component yield to be decreased. Therefore, the first goal for this thesis research is to bridge the knowledge gap by choosing a lower inclusion rate of MCFA to minimize the negative effects on rumen fermentation observed in previous studies and to understand energy and nutrient utilization during MCFA supplementation.

2. Rumen microbial diversity

When supplementing MCFA, previous research has measured total microbial populations in hopes to find a relationship between microbial populations and the observed decreases in methane emissions and NDF digestion. However, there is no study linking MCFA supplementation to changes in microbial diversity and species richness, which may help to better understand the relationship between the rumen microbiome and host. The second goal of this thesis is to identify changes in rumen microbiota when supplementing a low dose of MCFA.

The objective for this thesis were to evaluate effects of MCFA supplementation at < 1% of dietary DM on performance and plasma metabolite concentrations, apparent total tract nutrient digestibility, rumen fermentation and rumen microbial profile. I hypothesized that supplementation of MCFA at a lower dose (i.e., 0.05 to 0.2% of dietary DM) would increase rumen pH and milk production without decreasing nutrient digestibility which is typically observed with the higher inclusion rates (i.e., > 1% of dietary DM).

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Chapter 2. Effects of medium-chain fatty acid supplementation on performance and rumen fermentation of lactating dairy cows

2.1 Introduction

Medium-chain fatty acids (MCFA) are fatty acids with chain lengths between 6 – 12 carbons, and primarily metabolized by the liver but may also be absorbed and metabolized by intestinal mucosa (Guillot et al., 1994). As MCFA have a lower molecular weight and size compared to long chain fatty acids (LCFA), they are more soluble in biological liquids and absorbable without binding to a transporter (Hanczkowska, 2017). Compared with LCFA that requires the extended process for digestion, MCFA provide the animal with available energy more quickly because they are absorbed directly into the portal circulation and metabolized in the liver (Odle, 1997; Hanczkowska, 2017).

Medium-chain fatty acids have been shown to have antimicrobial properties and decrease the incidences of diarrhea in monogastric animals (Skrivanova et al., 2009; Chwen et al., 2013). Acidification in the gastric stomach may increase the antimicrobial effects of MCFA (Dierick et al., 2002; Zentek et al., 2011). At a low pH (i.e., 3-6), MCFA are in the undissociated form and become more lipophilic (Skrivanova and Marounek, 2007), which increases their diffusion across microbial cellular membranes, leading to successful inhibition of growth of *Escherichia coli* (Skrivanova et. al., 2009; Kim and Rhee, 2013) and *Salmonella* (Zentak et al., 2011).

In ruminants, these antimicrobial effects have shown to reduce in vitro methane production (Dohme et al., 2000). The reduction of methane emissions may be due to reduced fiber digestion by rumen bacteria and protozoa (Dohme et al., 2001; Machmuller et al., 2001). In addition, supplementation of MCFA at > 1% of DM decreases total populations of bacteria (Dohme et al,. 1999; Machmuller et al., 2001), archaea (Dohme et al., 1999; Dohme et al., 2001) and protozoa

(Dohme et al., 1999; Hristov et al., 2009; Faciola and Broderick, 2013). Patra and Yu (2013) reported that coconut oil, which is high in MCFA content, is more toxic to protozoa and fibrolytic bacteria due to the higher concentrations of MCFA. In addition, coconut oil may selectively inhibit certain ruminal archaea populations (Patra and Yu, 2013), suggesting that relative abundance of archaea species may alter methane production.

Machmuller (2006) suggested that supplementation of MCFA causes negative or positive effects in ruminants depending on its dosage and frequency. Several MCFA supplementation studies have focused on the mitigation of methane, therefore high doses, greater than 1% of dietary DM, have been tested (Dohme et al., 2000; Reveneau et al., 2012). Inclusion rates of MCFA at > 1% of DM have been successful at reducing methane emissions and abundance of protozoa associated with methanogenesis, but also decreased NDF digestibility when supplemented at 2.5% (Machmuller et al., 2001) or 5% of dietary DM (Dohme et al., 2001). In addition, Faciola and Broderick (2013) reported that feeding MCFA at 1 % of dietary DM decreased DMI, milk production, and nutrient digestibility. Furthermore, Reveneau et al. (2012) fed MCFA at 5% of dietary DM and reported that DMI and milk yield decreased.

However, several studies reported positive effects of MCFA supplementation at lower dosages (< 0.2 % of dietary DM). Piepers and De Vliegher (2013) observed immunomodulating effects to support neutrophil viability in early-lactation cows when supplementing MCFA at 25 g/d. In addition, supplementing MCFA at 0.186% of dietary DM increased milk yield (Souza et al., 2015a) and decreased the incidence of ketosis (Souza et al., 2015b). Although the mechanism accounting for these positive effects of MCFA is not known, a previous study reported that cows fed MCFA at 10 g/d had higher rumen pH (De Smet et al., 2009).

As such, we hypothesized that supplementation of MCFA at a lower dose (i.e., 0.05 to 0.2% of dietary DM) would increase rumen pH and milk production without decreasing nutrient digestibility which is typically observed with the higher inclusion rates (i.e., > 1% of dietary DM). The objectives of this study were to evaluate effects of MCFA supplementation at 0.063% of dietary DM on performance and plasma metabolite concentrations, apparent total tract nutrient digestibility, rumen fermentation and rumen microbial profile of mid-lactating dairy cows.

2.2 Materials and Methods

All experimental procedures used in this study were approved by the University of Alberta Animal Care and Use Committee: Livestock (AUP#3474) and conducted according to the guidelines of the Canadian Council on Animal Care (2009).

2.2.1 Animals, Diets and Experimental Design

Thirty (8 primiparous and 22 multiparous) Holstein cows in mid-lactation (637 ± 68.5 initial BW, 98.5 ± 27.4 DIM, mean ± SD) were used in a crossover design with two 28-d periods (University of Alberta, Dairy Research and Teaching Centre, Edmonton, AB). One week prior to the start of feeding experimental diets, all animals were fed a common lactating diet and baseline measurements of BW, BCS, DMI, milk yield and composition were obtained. Dietary adaptation occurred for 25 d and the final 3 d of each period were used for data and sample collection. Animals were assigned to treatment groups balanced for parity and daily milk production. Cows were fed a diet containing MCFA supplement at 0.25% of dietary DM (Biotica-C; CBS Bio-Platforms , Calgary, AB) or a control diet with dry ground corn replacing the MCFA supplement. The MCFA supplement contained 25% MCFA (consisting of 32% C8:0, 21% C10:0, and 47% C12:0) and 75% carrier ingredients (consisting of 63% ground corn, 11.5% silicon dioxide and 0.5% flavoring

component). The MCFA supplement was pre-mixed in the concentrate mix, stored in an outdoor grain bin, and mixed in TMR immediately before feeding. Diets were formulated to meet or exceed the recommendations of the dairy NRC for all nutrient requirements of an animal producing 36 kg/d of milk with 4.0% fat and 3.0% protein of milk composition (Table 1). All animals were housed in individual tie stalls and had free access to water. Cows were fed experimental diets as TMR once daily at 0730 h at 105 to 110% of actual feed intake of previous day. Cows were milked in their stalls twice daily at 0500 and 1600 h.

2.2.2 Data and Sample Collection

Data and samples were collected during the final three days of each period. The amount of feed offered, and refusals were recorded daily to calculate DMI. Feed ingredients were collected three consecutive days and composited to yield one sample per period. Dry matter of feed ingredients was determined in a forced air oven at 55°C for 48 h. The dried samples were ground in a Wiley Mill (Thomas Scientific, Philadelphia, PA) with a 1-mm screen.

Milk yield was recorded daily for all cows. Milk samples were collected from six consecutive milkings. Animal BW was recorded after morning milking and prior to feeding on the final two days of each period. Body condition score was measured by two individuals using a 5-point scale (Wildman et al., 1981) on the final two days of each period. Both BW and BCS measurements were averaged for each period before statistical analysis.

Blood samples were collected every 18 h (1300, 0700, 0100 and 1900 h) on the final three days of each period. All blood samples were obtained via the coccygeal vessel in evacuated tubes containing sodium heparin (Fisher Scientific Company, Nepean, ON, Canada), and the tubes were immediately placed on ice and centrifuged at 3,000 *g* (20 min, 4 °C). Plasma were collected from

supernatant and stored at -20 °C until analysis. Samples were combined in equal volume to form one representative sample for each cow per period.

Fecal samples were obtained every 9 h (1400, 2300, 0800, 1700, 0200, 1100, 2000, and 0500 h) and composited to make one sample per cow per period accounting for diurnal variation. Fecal samples were composited for each cow during the collection periods, dried in a forced air oven for 72 h at 55°C. Fecal samples were ground to pass through a 1-mm screen (Wiley Mill, Thomas Scientific).

A sub-set of nine ruminally-cannulated cows were used to measure rumen fermentation and microbiota. Rumen fluid was collected every 9 h (1400, 2300, 0800, 1700, 0200, 1100, 2000, and 0500 h) on the final three days of each period. Rumen fluid was composited by cow to create a representative sample accounting for every 3 h of a 24-h period. Rumen fluid was obtained from the cranial dorsal, cranial ventral, central, caudal dorsal, and caudal ventral sacs, and strained through a perforated material (Weed block; Easy Gardener Inc., Wako, TX). Samples were centrifuged at 3,000 *g* (20 min at 4 °C) immediately after collection and stored at -20 °C. Rumen digesta was collected two hours prior and four hours after feeding on the final day of each period. Approximately 50 mL of digesta was collected from each of the five locations in the rumen and immediately frozen on dry ice, then stored at -80°C until analysis. Rumen pH was recorded every 30 s continuously for 72 h (starting 1 hour prior to feeding) using an LRC logger inserted though the rumen cannula as described in Penner et al. (2006). Daily minimum, mean and maximum rumen pH along with duration and area below pH 5.8 were calculated daily for each cow and averaged for each period.

2.2.3 Sample Analysis

Feed and fecal samples were sent to Cumberland Valley Analytical Services (Hagerstown, MD), and analyzed for DM (AOAC International, 2002; method 930.15), OM (AOAC International, 2002; method 942.05), NDF (Van Soest et al., 1991), starch (Hall, 2009), CP (AOAC International, 2000; method 990.03), ether extract (EE) (AOAC International, 2006; method 2003.05). Indigestible NDF was determined after 240 h of in vitro digestion using ruminally cannulated cows, and used as an internal marker to estimate fecal output (Cochran et al., 1986). Apparent total tract digestibility of DM, OM, CP, NDF, EE and starch were determined.

Milk samples were analyzed for concentrations of fat, crude protein, lactose, SCC and MUN by infrared spectroscopy (AOAC International, 2002; method 972.16; MilkoScan 605, Foss North America, Brampton, ON, Canada) at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada). The 3.5% FCM was calculated as 0.432 × milk yield +16.23 × fat yield.

Plasma were analyzed using commercial kits for glucose (Autokit Glucose; Wako Chemicals USA Inc., Richmond, VA), nonesterified fatty acids (NEFA HR2; Wako Chemicals USA Inc., Richmond, VA), and insulin (Bovine Insulin ELISA, 10-1201-01; Mercodia, Uppsala, Sweden).

Gas chromatography was used for VFA analysis with methods adapted from Erwin et al. (1961). Briefly, rumen fluid was prepared for analysis by adding one fourth of total volume of 25% phosphoric acid, centrifuged at 13,000 g for 5 min at 4 °C, 200 μL of internal standard was added and allowed to incubate overnight at -20 °C. The internal standard was prepared by adding 20 mL of 25% phosphoric acid with 20 mL water, and 300 μL of isocaproic acid, brought to a total volume of 100 mL with water and mixed. Samples were placed on an autosampler, ran through a Varian 430GC (Agilent Technologies, Santa Clara, CA, USA) with split/split-less injector with a

Stabilwax column (30 m × 0.53 mm i.d.; Restek, Bellefonte, PA, USA) Injector temperature was 250 °C, initial column temperature was 80 °C, increased to 180 °C at 20 °C/min and held for 3 min and total run time is 8 min. Flame ionization detector temperature was held at 250 °C. Detector gas were set to the following settings: makeup flow = 20 mL/min, H flow = 30 mL/min, and air flow = 300 mL/min.

Total genomic DNA was extracted from approximately 0.7 g of rumen digesta using the repeated bead-beating and column method described in Yu and Morrison (2004). Genomic DNA was precipitated using 10M ammonium acetate and iso-propanol followed by purification using QIAamp Fast DNA Stool Mini Kit (QIAGEN Inc. CA, USA.). Quality and quantity of extracted DNA was assessed with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Paired-end sequencing of amplicon DNA was performed using the Illumina MiSeq PE300 at Genome Quebec at McGill University (Montreal, QC, Canada). Briefly, the amplification of V1-V3 region of bacterial 16S rRNA gene, protozoan 18S rRNA gene and V6-V8 region of archaeal 16S rRNA gene were performed using BAC9F (5'-GAGTTTGATCMTGGCTCAG) and 515R (5'-CCGCGGCKGCTGGCAC) primers, RP841F (5'-GACTAGGGATTGGARTGG) and REG1302R (5'-AATTGCAAAGATCTATCCC) primers, and Arc915aF (5'-AGGAATTGGCGGGGGAGCAC) and 1386R (5'- GCGGTGTGTGCAAGGAGC) primers, respectively (Henderson et al., 2015).

Sequencing data analysis were conducted using Quantitative Insight Into Microbial Ecology 2 (QIIME2; 2019.10 version) (Bolyen et al. 2019). Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with DADA2 (Callahan et al. 2016). Amplicon sequence variants (ASV) were aligned with mafft (Katoch et al.

2002) and was classified using the q2-feature classifier (Bokulich et al. 2018), classify-sklearn naïve Bayes taxonomy classifier with the SILVA 132 Small Subunit rRNA Database (Quast et al., 2013 and Yilmaz et al., 2014) for bacteria and protozoa, and the Rumen and Intestinal Methanogen-DB (RIM-DB) for archaea (Seedorf et al., 2014). Further microbial profile analysis including Alpha diversity (the Chao1 and Shannon indices) and Beta diversity (based on the Bray-Curtis index) were performed using MicrobiomeAnalyst (Dhariwal et al., 2017; Chong et al., 2020).

2.2.4 Statistical Analysis

Data from one ruminally-cannulated cow were removed due to various health issues throughout the study. Statistical analysis of DMI, milk yield, milk components, milk energy, digestibility, plasma metabolites and insulin were conducted using the Fit Model procedure of JMP (version 14, SAS Institute Inc., Cary, NC) with the following model:

$$Y_{ijkl} = \mu + T_i + P_j + A_k + C_l(A_k) + A_k*T_i + e_{ijkl},$$

where μ is the overall mean, T_i is the fixed effect of treatment ($i = 1$ to 2), P_j is the fixed effect of period ($j = 1$ to 2), A_k is the fixed effect of parity category ($k = 1$ to 2), $C_l(A_k)$ is the random effect of cow nested in parity ($k = 1$ to 29), A_k*T_i is the interaction of parity on treatment, and e_{ijkl} is the residual error. Significance was declared when $P < 0.05$ and tendencies were discussed when $P < 0.10$. Interaction between parity and treatment was included in the statistical model, however, results will not be shown or discussed as it was not significant for primary response variables.

Statistical analysis of rumen pH, VFA profile and Methanobrevibacter ratio were conducted using the Fit Model procedure of JMP (version 14, SAS Institute Inc., Cary, NC) with the following model:

$$Y_{ijkl} = \mu + T_i + P_j + C_l + e_{ijkl},$$

where μ is the overall mean, T_i is the fixed effect of treatment (i = 1 to 2), P_j is the fixed effect of period (j = 1 to 2), C_l is the random effect of cow (l = 1 to 8), and e_{ijl} is the residual error. Significance was declared when $P < 0.05$ and tendencies were discussed when $P < 0.10$.

Statistical analysis of rumen microbiota was conducted using R (version 4.0.2) within MicrobiomeAnalyst (Dhariwal et al., 2017). Alpha diversity was compared using the Mann-Whitney method, beta diversity was compared using Permutational MANOVA (PERMANOVA) and visualized using Principal Coordinate Analysis (PCoA). Sparse High- Throughput Sequencing Data (metagenomeSeq) was used to determine differences in ASV using a zero-inflated Gaussian fit model using local false discovery rate (FDR) adjusted P value (P_{adj}) of 0.05 to determine significance and P_{adj} between 0.05 and 0.1 were considered tendencies.

2.2.5 Data availability

All of the sequence data has been submitted to NCBI SRA under bioproject PRJNA733676.

2.3 Results and Discussion

2.3.1 DMI and Digestibility

Dry matter intake was not affected by MCFA supplementation ($P = 0.63$; Table 3). In addition, MCFA supplementation did not affect apparent total tract digestibility of DM ($P = 0.46$), OM ($P = 0.90$), NDF ($P = 0.32$), CP ($P = 0.53$), starch ($P = 0.20$), and ether extracts ($P = 0.30$). Our results are not consistent with previous research that reported decreases in DMI and NDF digestibility (Hollman et al., 2012; Faciola and Broderick, 2013; Fukumori et al., 2013).

Feeding MCFA may cause different effects on digestibility depending on the chain length. Dohme et al. (2001) evaluated effects of in vitro inclusion of C8:0 (caprylic acid), C10:0 (capric acid) or C12:0 (lauric acid) at 5% of DM and observed reductions in NDF digestion by 2.4%, 6.0% and 8.5% as compared to the control, respectively. This may be due to C12:0 having greater antimicrobial effects on bacterial cell growth and membrane integrity as compared to C8:0 and C10:0 (Shilling et al., 2013). In the current study, the MCFA supplement FA profile was 32% C8:0, 21% C10:0 and 47% C12:0. Faciola and Broderick (2013) supplemented C12:0 at 1% of dietary DM and observed a decrease in NDF digestibility. Hollmann et al. (2012) supplemented coconut oil at 1.3% of dietary DM which contained 9.1% C8:0, 6.7% C10:0 and 40.8% C12:0, and observed a reduction in NDF digestibility. However, Hristov et al. (2009) supplemented coconut oil at 0.96% of dietary DM which contained 7.0% C8:0, 6.1% C10:0 and 46.0% C12:0, and observed no change in NDF digestibility. Dohme et al. (2001) suggested the higher inclusion of C12:0 has greater reductions in NDF digestibility. However, MCFA evaluated in both Hollmann et al. (2012) and the current study were high in C12:0, but we observed no treatment effects on NDF digestibility while Hollmann et al. (2012) did. Therefore, type of MCFA may not explain the discrepancies between our study and previous studies in the literature.

Another possible explanation for the discrepancy in nutrient digestibility is the difference in inclusion rate of MCFA supplements among studies. In the current study, an MCFA supplement, containing 25% MCFA with 75% of carrier ingredient, was fed at 0.25% of dietary DM. Therefore, the inclusion rate of MCFA was 0.063% of dietary DM, and this may explain why we observed no differences in DM, OM or NDF digestibility. Dohme et al. (2001) supplemented pure caprylic acid, capric acid or lauric acid at 5% of dietary DM in vitro and

observed a reduction in NDF digestibility. Faciola and Broderick (2013) supplemented pure lauric acid at 0.97%, 1.95% and 2.92% of dietary DM and observed a linear decrease in DM, OM, and NDF digestibility. Hollman et al. (2012) supplemented coconut oil at 1.3%, 2.7% and 3.3% of dietary DM that contained 56.6% MCFA, which means overall dietary inclusions of MCFA at 0.73%, 1.52% and 1.87% DM, respectively, and observed a linear reduction in DM, OM and NDF digestibility. The response in NDF digestibility may be due to reduction to rumen protozoa populations as Faciola and Broderick (2013) reported a linear reduction of protozoa cells present in the rumen at higher inclusions of MCFA. William and Coleman (1997) estimated that protozoa may contribute up to one-third of the total NDF digestion in the rumen. However, it is hypothesized that protozoa contribute more to NDF digestion than the bacteria that replace them after defaunation (Firkins and Yu, 2006). Decreased NDF digestibility at 5% of dietary DM (Dohme et al., 2001) and the linear decrease in digestibility of DM, OM and NDF observed in previous research (Hollman et al., 2012; Faciola and Broderick, 2013) confirm the dose dependent response to MCFA supplementation in digestibility. Supplementation of MCFA between at 0.062% and 0.88% (Hristov et al., 2011) of dietary DM did not affect DM, OM or NDF digestibility. Therefore, we speculated that supplementation of MCFA at a lower dosage would not decrease nutrient digestibility.

In the current study, DMI was not affected by the MCFA supplement, however previous research had reported decreases in DMI when supplementing coconut oil (Hollmann et al., 2012), lauric acid (Faciola and Broderick, 2013), or MCFA calcium salts (Fukumori et al., 2013). The decrease in DMI caused by MCFA may be linked to decrease in NDF digestibility. Feed intake and NDF digestibility were observed to decrease linearly in both Hollmann et al. (2012) and Faciola and Broderick (2013). The decrease in DMI reported in the previous studies may be due

to the decreased rumen NDF digestion and decreased particle passage rate from the rumen, as observed by Reveneau et al (2012), leading to satiety caused by rumen fill (Allen, 2000). However, Hristov et al. (2011) observed a reduction in DMI but not in NDF digestibility, indicating that rumen fill is not the only reason for low DMI that is potentially caused by MCFA supplementation. Another possible explanation is that the rapid and unregulated oxidation of MCFA in the liver (McGarry et al., 1977; Dias et al., 1990) may induce sooner metabolic satiety via hepatic oxidation, decreasing DMI (Allen et al., 2009).

2.3.2 Rumen pH and VFA

The MCFA supplementation did not affect total VFA concentration ($P = 0.63$), and molar proportions (mol/100 mol of VFA) of acetate ($P = 0.87$), propionate ($P = 0.86$), and butyrate ($P = 0.72$). Acetate to propionate ratio did not differ between treatment groups ($P = 0.79$). However, minimum rumen pH tended to be higher when supplementing MCFA (5.66 vs. 5.54; $P = 0.08$) although mean rumen pH did not differ ($P = 0.89$). No difference was observed in maximum pH between treatment groups ($P = 0.15$), but daily fluctuation range of rumen pH decreased when supplementing MCFA (1.17 vs. 1.40; $P = 0.02$). No differences were observed in duration (min/d; $P = 0.12$) and area (pH × min/d; $P = 0.11$) of pH below 5.8.

The lack of treatment effects on mean rumen pH in our study is consistent with previous research (Hristov et al, 2004; Reveneau et al., 2012; Faciola and Broderick, 2013). However, we were able to observe that MCFA supplementation decreased diurnal fluctuation in rumen pH as we measured rumen pH every 30 s continuously for 3-d periods while previous studies had measured rumen pH at specific time points relative to feeding (Hristov et al, 2004; Faciola and Broderick, 2013) or at the end of the sampling period (Reveneau et al., 2012). The reduction in range of rumen pH when supplementing MCFA may allow for more consistent energy supply from

the rumen to blood circulation throughout a feeding period (Oba and Allen, 2000) as VFA absorption by rumen epithelium is partly regulated by rumen pH (Allen, 1997). These results suggest that MCFA supplementation, even at a low dosage (e.g., 0.063% of dietary DM evaluated in the current study), can affect rumen pH possibly due to changes in rumen microbiota (Dohme et al., 1999).

2.3.3 Rumen Microbiota

In total, an average of 23,682 ± 1,635 (mean ± SD) bacterial reads, 1,802 ± 388 archaeal reads, and 1,230 ± 211 protozoan reads per sample were obtained from the entire sample set. The predominant bacteria phylotype (% of bacteria) include unidentified genus of *Prevotellaceae* (12.3 ± 0.8%); unidentified genus of *Muribaculaceae* (10.9 ± 1.2%); unidentified genus of *Christensenellaceae* (6.9 ± 0.9%); unidentified genus of *Lachnospiraceae* (5.8 ± 1.2%); unidentified genus of *Clostridiales* (5.6 ± 0.9%); the predominant archaeal phylotype (% of archaea) include *Methanobrevibacter ruminantium* (86.8 ± 1.7%); *M. gottschalkii* (9.1 ± 3.1%); and the predominant protozoal phylotype (% of protozoa) include *Isotricha* (34.2 ± 4.4%); *Ophryoscolex* (30.5 ± 6.0%).

The MCFA supplementation tended to decrease Chao1 index of bacterial community at phylum level (10.9 vs. 11.6; $P = 0.07$; Table 5) but did not affect the Shannon index ($P = 0.80$). Chao1 estimates species richness whereas the Shannon index estimates species diversity, suggesting that species richness tends to be reduced by MCFA at the bacterial phylum level. As lower microbiome richness is related to higher feed efficiency (Shabat et al., 2016; Li and Guan, 2017), effects of MCFA supplementation on feed efficiency warrant further investigation. The MCFA treatment did not affect alpha diversity indices of bacteria at the family and genus levels ($P > 0.15$), Chao1 ($P = 1.00$) and Shannon ($P = 0.72$) of archaeal community at species level, and

Chao1 (P = 0.67) and Shannon (P = 0.44) of protozoa species. In addition, beta diversity of bacterial (P > 0.30; Figure 1), archaeal (P = 0.63), and protozoan communities (P = 0.91) were not affected by MCFA supplementation.

The MCFA treatment increased relative abundance of an unidentified family of *Mollicutes* (0.04 vs 0.01%; P_{adj} = 0.02; Table 6) and tended to increase relative abundance of an uncultured genus of *Bacteroidetes* (0.48 vs. 0.37%; P_{adj} = 0.06) although it did not affect relative abundance of fibrolytic bacteria, *Fibrobacter* (P_{adj} = 0.96) and *Ruminococcus* (P_{adj} = 0.96). For the archaeal community, MCFA supplementation increased relative abundance (% of total archaeal abundance) of *Methanobrevibacter gottschalkii* (5.14 vs. 4.92%; P_{adj} = 0.03) and decreased that of *Methanosphaera sp. ISO3-F5* (1.78 vs. 2.44%; P_{adj} = 0.06), but did not affect that of *M. ruminantium* (P_{adj} = 0.34). However, MCFA treatment did not affect relative abundance of all protozoa at genus level in this study (P = 0.94).

The tendency of higher minimum rumen pH in cows supplemented with MCFA may have caused the increase in relative abundance of unidentified family of bacteria from the *Mollicutes* class and uncultured bacterium that belongs to the *Bacteroidetes* phylum. Galbraith and Miller (1973) reported that lauric acid had greater antimicrobial activity against the *Mollicutes* class at a lower pH. However, increasing relative abundance of *Mollicutes* may not benefit ruminants as they are considered commensal or parasitic bacteria (Vega-Orellana et al., 2017). Increasing rumen Mollicutes also increases the risk of animals contracting contagious bovine pleuropneumonia, (OIE, 2020). Additionally, Mao et al. (2013) reported that subacute rumen acidosis could lead to the death and lysis of Bacteroidetes, further suggesting that increasing rumen pH may increase bacterium taxon relative abundance.

Previous research reported that MCFA supplementation reduced methane production in vivo (Hristov et al., 2009) and in vitro (Dohme et al., 1999). In the current study, we observed a decrease in *M. gottschalkii* and an increase in *Methanosphaera sp. ISO3-F5* which is consistent with Hristov et al. (2012) who evaluated effects of lauric acid supplementation. Kittelmann et al. (2013) reported a negative correlation between relative abundance of *M. gottschalkii* and *M. ruminantium*, and Danielsson et al. (2017) found that methane emissions increased as the ratio of *M. gottschalkii*:*M. ruminantium* increased. In the current study, MCFA supplementation increased relative abundance of *M. gottschalkii*, but did not affect *M. gottschalkii:M. ruminantium* ratio ($P = 0.99$; the ratio was normally distributed according to Shapiro-Wilk test, $P = 0.21$), and its effects on ruminal methane concentrations are not known. However, rumen methanogenesis is inhibited at pH lower than 6.5 (Van Kessel and Russel, 1996), and minimum rumen pH tended to be higher when supplementing MCFA in the current study. These results suggest that methanogenesis could potentially be higher for cows fed MCFA at a lower amount (i.e., 0.25% of dietary DM) due to the tendency for higher minimum rumen pH although the *M. gottschalkii:M. ruminantium* ratio was not affected.

2.3.4 Nutrient Utilization

The MCFA supplementation did not affect plasma concentrations of glucose ($P = 0.55$; Table 7), free fatty acids ($P = 0.18$), and insulin ($P = 0.87$). In addition, MCFA supplementation did not affect yields of milk ($P = 0.31$; Table 8), milk fat ($P = 0.43$), milk protein ($P = 0.29$), lactose ($P = 0.40$), and 3.5% FCM ($P = 0.29$), and milk urea nitrogen concentration ($P = 0.28$). In addition, changes in BW and BCS did not differ between treatment groups ($P = 0.49$).

In the current study, overall animal performance was not affected by MCFA supplementation, but pretrial milk yield was negatively related with animal responses to MCFA

treatment in milk protein yield; MCFA treatment decreased milk protein yield to a greater extent in higher producing cows (P = 0.04; r = -0.38; Figure 2). Previous research reported that MCFA supplementation decreased milk protein yield (Hollman et al., 2012; Faciola and Broderick, 2013; Fukumori et al., 2013). Faciola and Broderick (2013) reported a linear decrease in protozoa population when supplementing lauric acid along with a decrease in rumen ammonia and free amino acids. A reduction in rumen protozoal count due to MCFA supplementation (Newbold and Chamberlain, 1988; Matsumoto et al., 1991), associated with decreased ammonia concentration (Williams and Coleman, 1992), is most likely a result of a reduction in protozoal breakdown of bacterial proteins (Broderick et al., 1991). The combination of a reduction in microbial protein and rumen ammonia concentration may account for the reduction of milk protein yield in lactating ruminants when supplementing MCFA (Hristov and Ropp, 2003). In the current study, no differences were observed in rumen protozoa relative abundance. However, reduced milk protein yield with the MCFA treatment for higher producing cows who consumed more MCFA from greater DMI is consistent with expected reductions in microbial protein production in the rumen.

Pretrial milk yield was also negatively related with animal responses to the MCFA treatment in BW change; higher producing cows tended to increase BW to a less extent when MCFA is supplemented (P = 0.06; r = -0.35; Figure 3). Medium-chain fatty acids have been shown to reduce body fat in rats (Baba et al., 1982; Han et al., 2003). Han et al. (2003) supplemented MCFA, consisting of C8:0 and C10:0, to rats at 19.4% of the diet as fed and reported a downregulation of adipogenic genes. Baba et al. (1982) supplemented MCFA at 25% of the diet as fed and reported a decrease in adipose cell size. Stromal vascular cells from subcutaneous adipose tissue of dairy cattle decreased in leptin expression when treated with 1 mM of caprylate

(Soliman et al., 2007). An increase in adipose tissue prepartum has been associated with greater insulin resistance and metabolic disorders during early lactation (Holtenius and Holtenius, 2007), suggesting that reducing obesity in mid-lactation may be beneficial to the health of dairy cows during the transition period. Potentially, as high-producing cows have greater DMI, the increased dietary MCFA were able to decrease adipose tissue growth. However, body weight and BCS was not affected by MCFA in the current study.

2.4 Conclusion

Inclusion of MCFA in the diets of mid-lactation dairy cows at 0.063% of dietary DM did not affect milk yield, DMI and total tract nutrient digestibility. However, MCFA supplementation decreased the range of rumen pH and tended to increase minimum rumen pH. Rumen alpha and beta diversity of bacteria, archaea and protozoa were not affected by MCFA supplementation, but relative abundance of *M. gottschalkii* increased and *Methanosphaera sp. ISO3-F5* decreased. These results warrant further research on the relationship among rumen microbiome, rumen pH, and methane emissions when MCFA are supplemented in the diet of lactating dairy cows.

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2.7 Tables and Figures

Table 2.1 Animal BW, BCS, parity, DMI, milk yield and components during the baseline period when fed a common diet (n = 30)

Variable	Mean	± SD[1]
BW, kg	637	68.1
BCS	2.60	0.20
Parity	2.53	1.28
DMI, kg/d	26.4	2.90
Milk yield, kg/d	39.3	7.12
Fat yield, kg/d	1.35	0.23
Protein yield, kg/d	1.18	0.18
Lactose yield, kg/d	1.80	0.32
Fat content, %	3.47	0.40
Protein content, %	3.03	0.20
Lactose content, %	4.59	0.16

[1]SD = standard deviation

Table 2.2 Ingredient and chemical composition of experimental diets

	Treatments[1]	
Item	CON	MCFA
Ingredients, % of DM		
Barley silage[2]	38.9	38.9
Alfalfa hay[3]	6.7	6.7
Dry ground barley grain[4]	16.8	16.8
Dry ground corn grain[4]	15.3	15.3
Corn gluten meal	3.1	3.1
Canola meal	7.5	7.5
Soyhulls	1.5	1.5
Bypass soy[5]	5.6	5.6
Palm fat[6]	1.8	1.8
Limestone	1.0	1.0
Magnesium oxide	0.2	0.2
Sodium bicarbonate	0.6	0.6
Red salt	0.4	0.4
Mineral premix[7]	0.6	0.6
Trace mineral/vitamin premix[8]	0.2	0.2
Biotica – C[1]	-	0.25
Dry ground corn grain	0.25	-
Chemical Composition, % of DM		
DM	45.1	45.2
CP	18.6	18.6
NDF	31.0	31.0
ADF	20.6	20.3
Starch	26.4	26.3
Ether extract[9]	4.6	4.4
Ash	8.9	8.9
NE_L, Mcal/kg of DM[10]	1.59	1.59
ME allowable milk, kg/d[10]	36.1	36.1
MP allowable milk, kg/d[10]	39.1	39.1

[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM. Supplement contained 25% MCFA and 75% carrier. Total inclusion of MCFA is 0.063% of dietary DM (32% C8:0, 21% C10:0 and 47% C12:0) in a carrier mixture of 63% ground corn, 11.5% silicon dioxide and 0.5% flavoring component.
[2] 23.9% DM, 55.9% NDF, 13.6% CP, 7.05% starch, 2.91% EE (on a DM basis except for DM).
[3] 92.4% DM, 38.6% NDF, 21.4% CP, 1.05% starch, 1.50% EE (on a DM basis except for DM).
[4] Ground using a hummer mill with 0.32-cm screen
[5] Soyplus, Landus, Ames, IA, United States
[6] Jefo, Saint-Hyacinthe, QB, Canada
[7] Contained 22% calcium and 12% magnesium

[8] Contained commercial product Availa Plus (Zinpro, Eden Prairie, MN), 2,535 mg/kg Fe, 31,350 mg/kg Zn, 5,000 mg/kg Cu, 26,500 mg/kg Mn, 523.28 mg/kg Co, 440 mg/kg I, 500 mg/kg F, 142 mg/kg Se, 3,115,000 IU/kg vitamin A, 733,00 IU/kg vitamin D3, 22,220 IU/kg vitamin E

[9] Standard deviation of EE is 0.156%

[10] Estimated from NDS, CNCPS 6.55 (RUM&N, Reggino Emilia, Italy)

Table 2.3 Effect of MCFA on nutrient intake and apparent total tract DM, OM, NDF, Starch and EE digestibility in mid-lactation dairy cows (n = 29 per treatment)

	Treatments[1]		SEM	P-value
Variable	CON	MCFA		
DMI, kg/d	24.0	24.3	0.62	0.63
Digestibility, %				
DM	70.5	70.3	0.28	0.46
OM	70.5	70.5	0.28	0.90
NDF	44.7	45.2	0.50	0.32
CP	74.5	74.2	0.39	0.53
Starch	99.4	99.3	0.07	0.20
Ether extracts	84.4	83.1	0.96	0.30

[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM

Table 2.4 Effects of MCFA supplement on rumen pH and volatile fatty acids of multiparous mid-lactation dairy cows (n = 8 per treatment)

Variable	Treatments[1]		SEM	P-value
	CON	MCFA		
Total VFA, m*M*	148	146	1.97	0.63
VFA profile, mol/100 mol of VFA				
Acetate	64.2	64.1	0.41	0.87
Propionate	20.1	20.2	0.41	0.86
Butyrate	10.8	10.6	0.30	0.72
Valerate	1.61	1.65	0.03	0.26
Isobutyrate	0.95	0.96	0.01	0.61
Isovalerate	1.80	1.86	0.70	0.11
A/P ratio	3.21	3.18	0.08	0.79
Rumen pH				
Minimum	5.54	5.66	0.04	0.08
Mean	6.22	6.21	0.38	0.89
Maximum	6.95	6.83	0.05	0.15
Range	1.40	1.17	0.05	0.02
Duration pH <5.8, min/d	143.6	56.1	27.9	0.12
Area pH <5.8, pH x min/d	25.10	5.59	6.96	0.11

[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM

Table 2.5 Effects of MCFA supplement on alpha-diversity in the rumen of mid-lactation dairy cows (n = 8 per treatment)

	Treatments[1]		SEM	P-value
Variable	CON	MCFA		
Bacteria				
Phylum				
Chao1	11.6	10.9	0.19	0.07
Shannon	0.93	0.91	0.01	0.80
Family				
Chao1	40.3	39.9	0.47	0.63
Shannon	2.40	2.40	0.01	0.80
Genus				
Chao1	114	116	1.20	0.15
Shannon	3.50	3.18	0.02	0.80
Archaea				
Species				
Chao1	3.00	3.00	0.00	1.00
Shannon	0.48	0.47	0.04	0.72
Protozoa				
Species				
Chao1	5.25	5.63	0.34	0.67
Shannon	1.02	1.07	0.08	0.44
Good's Coverage				
Bacteria	>98%	>98%	-	-
Archaea	>99%	>99%	-	-
Protozoa	>99%	>99%	-	-

[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM

Table 2.6 Effects of MCFA supplementation on rumen ASV relative abundance in mid-lactation dairy cows (n = 8 per treatment)

ASV	Phylotype	Treatments[1]		P_{adj}[2]
		CON	MCFA	
	Bacteria			
	Phylum			
ASV00003	Bacteroidetes	19.6%	21.3%	0.61
ASV00007	Fibrobacteres	1.11%	0.89%	0.61
ASV00008	Firmicutes	27.5%	26.8%	0.67
ASV00016	Tenericutes	0.05%	0.07%	0.61
	Family			
ASV00023	Prevotellaceae	9.93%	10.6%	0.87
ASV00035	Fibrobacteraceae	1.11%	0.89%	0.87
ASV00048	Ruminococcaceae	7.45%	7.41%	0.95
ASV00083	Unidentified family of bacteria in the *Mollicutes* class	0.01%	0.04%	0.02
	Genus			
ASV00040	Prevotella 1	6.01%	6.33%	0.96
ASV00056	Uncultured bacterium in the *Bacteriodates* phylum	0.37%	0.48%	0.06
ASV00069	Fibrobacter	0.84%	1.16%	0.96
ASV00156	Ruminococcus 2	1.61%	1.38%	0.96
	Archaea			
	Species			
ASV00002	Gottschalkii	4.92%	5.14%	0.03
ASV00003	Ruminantium	46.1%	40.7%	0.34
ASV00004	Methanosphaera sp. ISO3-F5	2.44%	1.78%	0.03
	Protozoa			
	Genus			
ASV00002	Entodinium	14.5%	13.5%	0.94
ASV00003	Eremoplastron	0.62%	1.27%	0.94
ASV00004	Isotricha	17.0%	17.2%	0.94
ASV00006	Polyplastron	0.67%	1.17%	0.94

[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM

[2] P value adjusted for false discovery rate

Table 2.7 Effects of MCFA supplement on plasma metabolites of mid-lactation dairy cows (n = 29 per treatment)

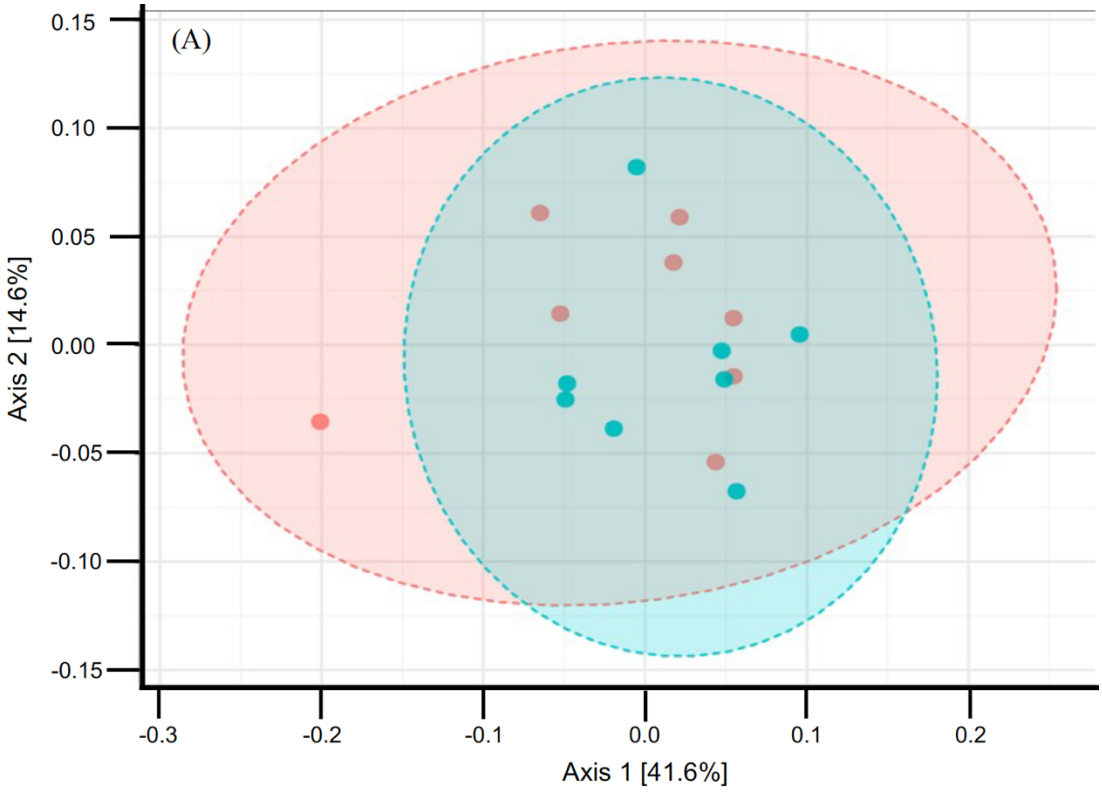
	Treatments[1]		SEM	P-value
Variable	CON	MCFA		
Glucose, mg/dL	72.4	73.1	0.97	0.55
Free fatty acids, μEq/L	155	147	5.8	0.18
Insulin, μg/L	6.35	6.31	0.19	0.87

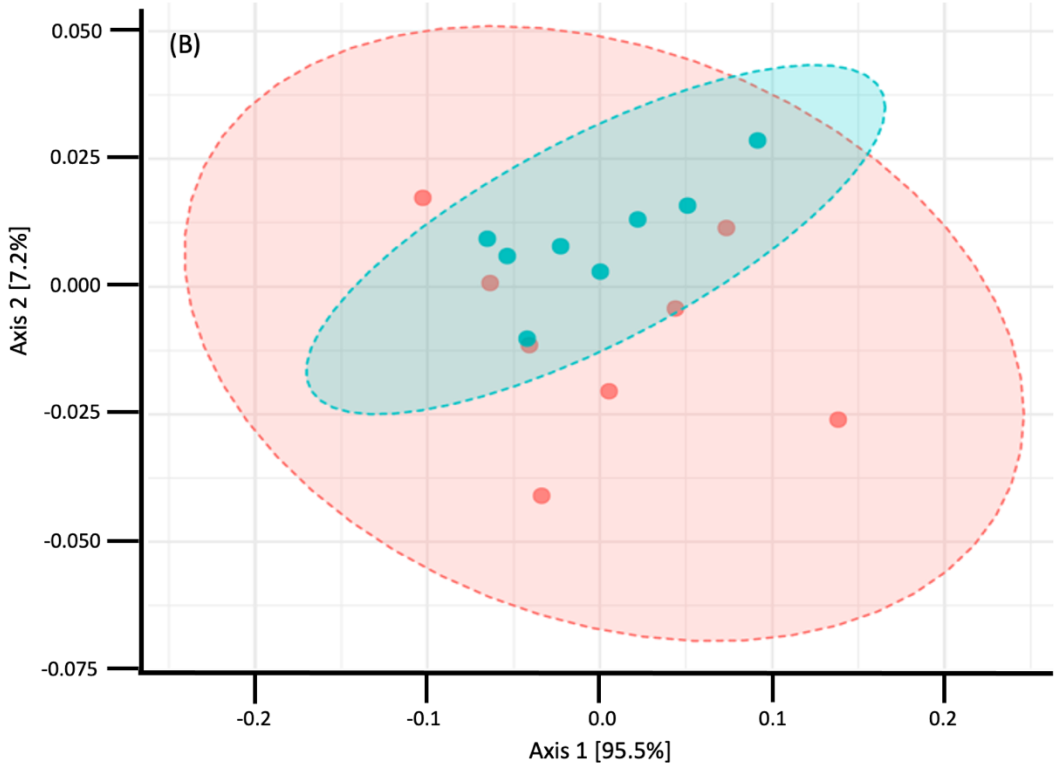
[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM

Table 2.8 Effect of MCFA supplement on DMI, BW and BCS changes, milk yield and composition of mid-lactation dairy cows (n = 29 per treatment)

Variable	Treatments[1]		SEM	P-value
	CON	MCFA		
Yield, kg/d				
Milk	36.4	36.9	0.86	0.31
Fat	1.43	1.45	0.04	0.43
Protein	1.23	1.19	0.03	0.29
Lactose	1.68	1.70	0.04	0.40
3.5% FCM	38.9	39.5	0.85	0.29
ECM	39.0	39.6	0.86	0.33
Composition				
Fat, %	3.98	3.99	0.09	0.92
Protein, %	3.31	3.32	0.04	0.40
Lactose, %	4.61	4.61	0.03	0.83
MUN, mg/dL	18.8	19.1	0.43	0.28
SCC, $\times 10^3$ cells/mL	83.6	91.4	28.0	0.65
Milk energy, Mcal/d	26.6	27.1	0.60	0.24
Milk/DMI	1.44	1.45	0.04	0.86
ECM/DMI	1.55	1.56	0.03	0.87
BW change, kg/d	0.52	0.65	0.11	0.49
BCS change per d	0.04	-0.01	0.02	0.13

[1] CON = control diet, MCFA = Biotica – C supplemented diet at 0.25% dietary DM


(A)
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
Axis 2 [14.6%]
-0.3
-0.2
-0.1
0.0
0.1
0.2
Axis 1 [41.6%]


(B)
0.050
0.025
0.000
-0.025
-0.050
-0.075
Axis 2 [7.2%]
-0.2
-0.1
0.0
0.1
0.2
Axis 1 [95.5%]

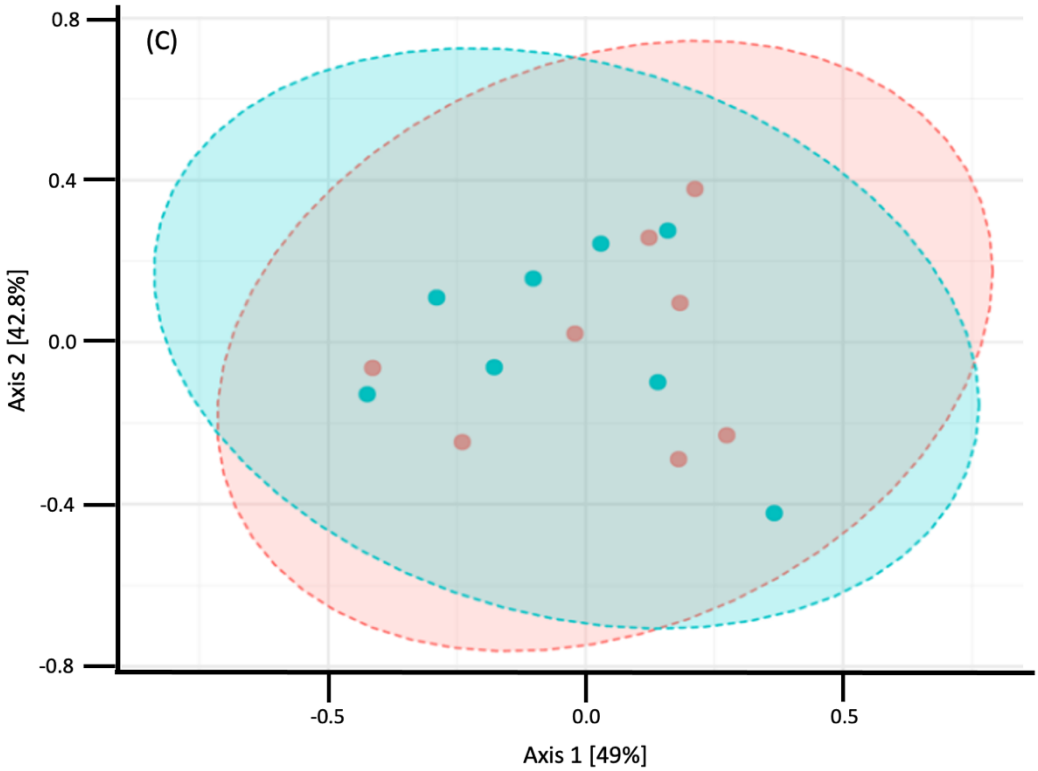


Figure 2.1 Effects of MCFA supplementation on beta-diversity of rumen microbiota with PCoA. Red and blue represent rumen microbiota of mid-lactation dairy cows fed either control or MCFA supplemented diets, respectively (n = 8). (A) Bacteria genus profiles plotted against the first two principal component axis (PC1 and PC2), which explain 41.6% and 14.6% of the variance (P = 0.45). (B) Archaea species profiles plotted against the first two principal component axis (PC1 and PC2), which explain 95.5% and 7.2% of the variation (P = 0.63). (C) Protozoa species profiles plotted against the first two principal component axis (PC1 and PC2), which explain 49% and 42.8% of the variance (P = 0.91).

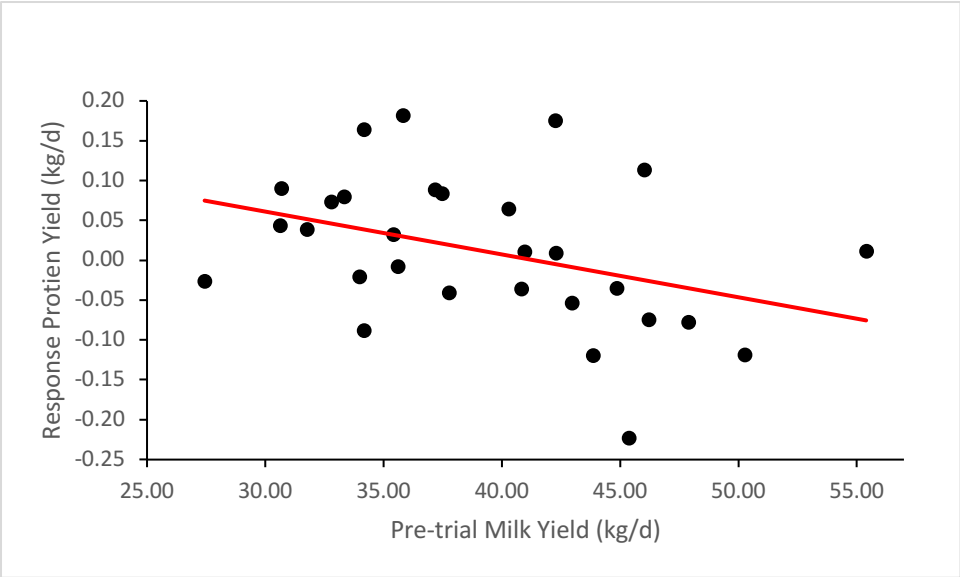


Figure 2.2 Relationship between average milk yield over 3 d prior to beginning of experiment and response in protein yield to MCFA treatment (MCFA – CON). High-producing cows tend to decrease protein yield to a greater extent (P = 0.04; r = -0.38) when MCFA is supplemented.

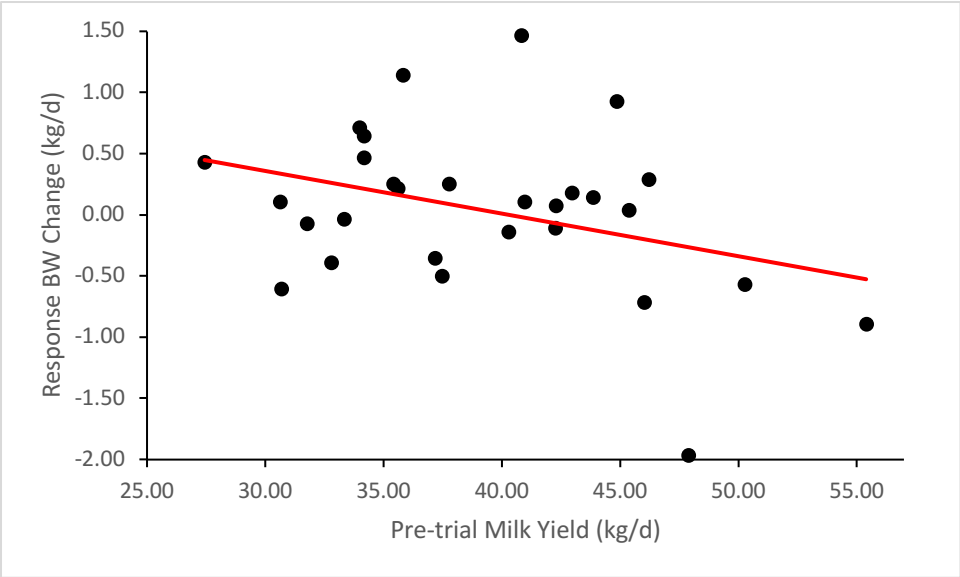


Figure 2.3 Relationship between average milk yield over 3 d prior to beginning of experiment and response in body weight change to MCFA treatment (MCFA – CON). High-producing cows tend to increase BW to a less extent (P = 0.06; r = -0.35) when MCFA is supplemented.

Chapter 3. General Discussion

3.1 Summary

Medium-chain fatty acid supplementation has been studied due to the rapid oxidation in the liver, providing available energy to the animal faster than LCFA. Ketones may be formed because of an excess of acetyl CoA from the β-oxidation of MCFA. Non-hepatic tissues utilize ketones as an energy source, thus sparing glucose for milk production (Schonfeld and Wojtczak, 2016). Additionally, the amphiphilic structure of MCFA allows for antimicrobial effects by intrusion through microbial membranes and inhibition of microbial enzymes; leading researchers to observe the effects on rumen fermentation and methanogenesis.

Many previous studies have supplemented MCFA at greater then 1% of dietary DM, resulting in successful reduction in methanogenesis. However, negative effects are observed on DMI, nutrient digestibility, milk yield and milk component yield, and decreasing the potential profitability (Hollmann et al., 2012; Faciola and Broderick, 2013). Chapter 2 evaluated the effects of MCFA supplementation in the diets of mid-lactation cows on animal performance and rumen fermentation. The MCFA supplement contained 25% FA and 75% carrier ingredient and fed at 0.25% of dietary DM. Therefore, the inclusion rate of MCFA was 0.063% of dietary DM. Dry ground corn was used in the control diets (CON) and replaced by MCFA in the treatment diet. All animals were fed a common diet for one week prior to beginning experimental diets to collect baseline performance measurements. Adaptation to experimental diets occurred for 25 days followed by 3 days of data and sample collections.

In my thesis study, supplementing MCFA at 0.063% of dietary DM did not affect DMI, successfully eliminating the biggest negative effect of MCFA observed in previous research (Hollmann et al., 2012; Faciola and Broderick, 2013; Fukumori et al., 2013). In Chapter 1, the

factors limiting DMI when supplementing MCFA was discussed and concluded that NDF digestibility has one of the biggest effects on DMI in lactating dairy cows. Supplementing MCFA, as shown in Chapter 2, did not affect NDF digestibility, along with OM, CP, starch, and ether extracts digestibility. Medium-chain fatty acid supplementation did not limit nutrients available for intestinal absorption, leading to no effects on milk yield and milk component yield.

In Chapter 2, it was found that MCFA supplementation had no effects on plasma glucose, FFA, and insulin concentrations. Furthermore, feed efficiency did not differ between treatment groups, suggesting that the utilization of nutrients was not altered by MCFA. A few interesting relationships were observed in the correlation between pretrial milk yield and animal response to MCFA supplementation. The first is that high-producing cows tended to decrease milk protein yield to a greater extend when MCFA is supplemented. High-producing cows have greater DMI, resulting in consuming more MCFA per day as compared to low-producing cows. Previous research suggested that MCFA linearly decrease rumen protozoa populations as inclusion of MCFA in the diet increases (Faciola and Broderick, 2013). Protozoa are three times more efficient in deaminating amino acids of bacteria origin (Broderick et al., 1991). It is likely that a decrease in protozoal populations occurred in high-producing cows, leading to a decrease in bacterial protein breakdown (Broderick et al., 1991) and intestinal flow of microbial proteins (Hristov and Ropp, 2003), decreasing milk protein yield observed in high-producing cows.

Additionally, it was observed that high-producing cows tended to increase body weight to a lesser extent when supplemented with MCFA. Previous research has shown that MCFA supplementation to rats is able to reduce body fat via downregulating the expression of adipogenic genes (Han et al., 2003) and decrease adipose cell size (Baba et al., 1982), suggesting that the increased daily intake of MCFA by high-producing cows may have influenced adipose

tissue metabolism to a greater extent. However, BW and BCS were not affected by MCFA supplementation in my study.

Furthermore, as shown in Chapter 2, total rumen VFA concentrations and VFA profile did not differ when MCFA were supplemented. Minimum rumen pH tended to be increased when supplementing MCFA, however mean and maximum rumen pH did not differ. Daily range of ruminal pH decreased when supplementing MCFA. It is important to note that the study in Chapter 2 is the first study to measure rumen pH continuously when supplementing MCFA, the mechanism in regulating rumen pH is unknown but may be attributed to the antimicrobial effects of MCFA. However, MCFA supplementation, at a low dosage that does not affect nutrient digestibility or DMI, can influence rumen fermentation.

Chapter 2 showed that bacterial phylum species richness tended to be reduced when MCFA were supplemented. The remainder of alpha diversity indices for bacterial family and genus, archaeal species and protozoal species were not affected by MCFA. Beta diversity of rumen microbiota was not affected by the MCFA supplement. Relative abundance of *M. gottschalkii* was increased by MCFA, suggesting the ratio of *M. gottschalkii:M Ruminatium* could have been increased (Kittelmann et al., 2013), however this ratio did not differ between treatment groups. In addition, relative abundance of *Methanosphaera sp.* decreased. Based on the changes in methane producing species when supplementing MCFA at 0.063% of diet DM, further research is warranted to understand methane emissions when feeding low doses of MCFA.

3.1.1 Industry implications

Based on the current research evidences, I would not suggest feeding MCFA to mid-lactation dairy cows. The MCFA supplement may increase the cost of feed, thus without a return

on investment in terms of milk yield or improved health, profitability per cow may decrease. As discussed in Chapter 1, supplementing MCFA at high doses exhibits a linear decrease in DMI, nutrient digestibility, milk and milk component yield. Any mistake during the feed mixing process, whether at a feed mill or on farm, can drastically and immediately cause negative effects on production.

There is potential for low inclusion rates of MCFA to have positive effects on rumen pH, such as mitigating sub-acute rumen acidosis (Marie Krause and Oetzel, 2006) and allowing for a more consistent energy supply to the host (Oba and Allen, 2000). Fresh cows may receive the most benefit from low dose supplementation of MCFA. As animals are switched from a high fiber diet during the dry period to a highly fermentable diet in the fresh period, risk of acidosis increases. Sub-acute rumen acidosis experienced by fresh cows may decrease DMI and milk yield (Abdela, 2016). Thus, by feeding MCFA, minimum rumen pH may be increased during the fresh period and ease the transition into lactation when feeding a highly fermentable diet. Preventing a decrease in DMI and milk yield during early lactation may allow for higher milk production at peak lactation and throughout the lactation.

3.2 Limitations

3.2.1 Dose Dependent Response

The study in Chapter 2, only one inclusion rate of MCFA (e.g., 0.063% of diet DM) was selected due to limited animal availability and funding. Medium-chain fatty acids exhibit a linear dose dependent response. The limitation of the study in Chapter 2 is that we can only make conclusions about animal performance and rumen fermentation when MCFA are supplemented

at 0.063% of dietary DM. Therefore, rumen fermentation and animal response to MCFA supplementation between 0.063% and 1% of dietary DM is unknown.

Previous studies have observed a linear decrease in DMI and nutrient digestibility when supplementing MCFA in TMR at greater than 0.97% of dietary DM (Hollmann et al., 2012; Faciola and Broderick, 2013). When MCFA are supplemented in TMR at 0.97% of dietary DM, DMI is reduced by 7% and protozoal populations are reduced by 28% (Faciola and Broderick, 2013). Whereas intraruminal infusion of MCFA at 0.88% of dietary DM, given as one pulse dose prior to feeding, reduced DMI by 25% and protozoal populations by 95% (Hristov et al., 2011). It has previously been suggested that the rate of MCFA inclusion in TMR must be much greater than the inclusion given as a single intraruminal infusion to elicit similar animal responses in DMI, rumen fermentation and milk yield (Faciola and Broderick, 2013). In theory, MCFA fed in TMR enter the rumen at a slower rate and the maximum concentration of MCFA in rumen digesta is likely lower than that of intraruminal infused MCFA given as a single dose (Hristov et al., 2009).

As reported in Chapter 2, MCFA supplementation did not affect DMI or nutrient digestibility. However, it was observed that minimum rumen pH tended to increase, and daily range of rumen pH was decreased. It is possible that supplementation of MCFA at 0.50% or 0.75% of dietary DM may not affect DMI, as observed at higher inclusion rates, but increase minimum rumen pH and decrease the daily range of rumen pH, as observed in Chapter 2.

3.2.2 Type of MCFA

In Chapter 2, caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0) were supplemented at 0.02%, 0.013% and 0.03% of dietary DM, respectively. We could not alter that ratio of FA supplied as the MCFA supplement was obtained from a commercial supplier. The

MCFA supplement used in this study was a mixture of FA and we could not evaluate the effects of specific MCFA. As each MCFA is expected to exert different effects, the MCFA supplement is one of the limitations in this study.

In previous studies, MCFA chain length has been observed to elicit varying *in vitro* responses. Dohme et al. (2001) observed a 2.4%, 6.0% and 8.5% decrease in NDF digestion when C8:0, C10:0 and C12:0 was supplemented, respectively. In addition, methane emissions increased by 20% and 12% when C8:0 and C10:0 were supplemented, respectively, whereas C12:0 decreased methane emissions by 11% (Dohme et al., 2001). Lauric acid (C12:0) has been shown to decrease bacterial, protozoal cell growth and decrease cell membrane to a greater extent compared to C8:0 and C10:0 (Shilling et al., 2013).

Due to the rapid oxidation of MCFA in the liver, MCFA may be incorporated into adipose tissue to a lesser extent compared to LCFA (Lemarie et al., 2016). Han et al. (2003) observed that supplementation of C8:0 and C10:0 to rats decreased body weight with downregulation of adipogenic genes compared to rats fed LCFA. In Chapter 2, we observed that high-producing cows gained body weight to a lesser extent when supplementing MCFA. Generally, high-producing cows have a greater DMI, thus increasing the daily amount of MCFA consumed. I speculate that high-producing cows may have prevented adipose tissue growth by increasing C8:0 and C10:0 intake.

3.2.3 Ruminally Cannulated Animals

The study in Chapter 2 relied upon eight ruminally cannulated cows in a crossover design for measurements of rumen VFA concentration, rumen pH and rumen microbiota. A small sample size may have reduced the opportunity to observe significant treatment effects. *P* value less than 0.05 was used to declare significance and *P* values between 0.10 and 0.05 was used to

declare tendency. In Chapter 2, we observed a tendency for minimum rumen pH to be increased by the MCFA supplement (5.66 vs. 5.54; $P = 0.08$). In addition, two rumen pH variables numerically decreased when supplementing MCFA: duration of pH < 5.8 (56.1 vs. 143.6; min/d; $P = 0.12$) and area pH < 5.8 (5.59 vs. 25.10; pH × min/d; $P = 0.11$). It is possible that with a higher sample size, differences in the previously mentioned pH variables may become significant. Sample size, bias, and random error are factors that may affect the statistical significance (Thiese et al., 2016). By increasing sample size, the likely hood of observing significance increases as random error decreases (Thiese et al., 2016).

3.2.4 Microbial Analysis

In Chapter 2, the microbial analysis did not include quantification of microbial populations. We were unable to determine if MCFA supplemented at 0.063% of dietary DM affected total microbial populations. Previous research has reported a decrease in total microbial populations when supplementing MCFA at > 1% of DM (Dohme et al., 2001; Faciola and Broderick, 2013). Utilizing qPCR techniques, total microbial population data would have been available; allowing us to compare results with previous research.

In addition, Stamatopoulou et al. (2020) found a number of MCFA-producing bacterial species in the rumen. In Chapter 2, MCFA produced by rumen microbes were not measured and they may have influenced rumen fermentation. *Eubacterium pyruvativorans* was identified in sheep rumen and are known to convert C3 and C4 volatile fatty acids to C5 and C6 by utilizing methanol and pyruvate as electron donors (Stamatopoulou et al., 2020). Methanol is a key substrate required for the survival and growth of Methanosphaera sp. (Friedman et al., 2017). In Chapter 2, the decrease in relative abundance of *Methanosphaera sp.* in animals fed MCFA

suggest rumen concentrations of methanol may have been decreased. In the current literature it is unknown if supplementing MCFA affects MCFA-producing bacteria.

3.3 Future Studies

3.3.1 MCFA to calves through colostrum and milk replacer

Medium-chain fatty acids in colostrum contribute approximately 5.25% of the total FA (O'Callaghan et al., 2020) and increase to approximately 11% by 30 days in milk (Thanh and Suksombat, 2015). This increase in MCFA from colostrum to milk may suggest, from an evolutionary perspective, that MCFA content in the diet is important in calf development. Jaster et al. (1992) suggested that when additional fat is provided in milk replacer, calf health is improved, and growth performance is increased. When supplementing a blend of short- and medium-chain fatty acids monoglycerides, cytoplasmic protrusion on the papillary tip increased suggesting increased epithelial growth and higher efficiency of absorption, however ADG did not differ between treatment groups (Ragionieri et a., 2016). In addition, when feeding approximately 40% of FA as MCT, Mills et al. (2010) observed no difference in DMI or ADG.

Additionally, the antimicrobial effects discussed in Chapter 1 may alter intestinal microbial establishment in pre-ruminant calves. The establishment of microbes in early life plays an important role in host intestinal barrier functions, metabolism, and health (Petersson et al., 2010). In pigs, supplementation of MCFA increased ileal and rectal counts of *Lactobacillus* (Kuang et al., 2015), which are beneficial in preventing diarrhea (Timmerman et al., 2005). No previous study has evaluated the establishment of intestinal microbiota when supplementing MCFA in colostrum or milk replacer of calves.

To test the effects of MCFA on calf growth, rumen development and intestinal microbiota establishment, colostrum and milk replacer could be supplemented with MCFA to

double the natural inclusion of MCFA in colostrum and milk (e.g., colostrum contains ~5.25% MCFA; supplemented colostrum would contain 10.5% MCFA. Milk contains ~11% MCFA; supplemented milk replacer would contain 22% MCFA). The MCFA supplemented colostrum would be fed for the first 24 h after birth, MCFA supplemented milk replacer will be fed from 24 h after birth until weaning. Due to the rapid oxidation of MCFA in the mitochondria of intestinal mucosa (Marten et al., 2006); I speculate that MCFA supplementation in colostrum and milk replacer will increase the digestibility of fluid milk and solid feeds by increasing the absorptive efficiency of the intestines. Medium-chain fatty acids may alter the establishment of intestinal microbiota thus preventing the prevalence of diarrhea in calves.

3.3.2 MCFA during the transition period

At the onset of lactation, dairy cows are switched from a low energy density diet to a high energy density diet. This is done through increasing the rapidly fermentable carbohydrate content of the diet. An increase in microbial VFA production occurs thus decreasing ruminal pH, often leading cows to experience sub-acute rumen acidosis (SARA). Sub-acute rumen acidosis poses many negative consequences in the productivity of dairy cows including decreased DMI (Plaizier et al., 2008), milk fat depression (Stone, 1999), and inflammation (Gozho et al., 2005). In addition, oxidative stress is positively related to diets high in grain or starch (Sgorlon et a., 2008). Research in rats and humans have shown that MCFA are able to scavenge for free radicals, preventing cell and tissue damage from oxidative stress (Henry et al., 2002; Lemieux et al., 2011).

Previous research has supplemented MCFA to transition cows, it was observed that milk production did not differ compared to the control, however, MCFA had a positive effect on cellular immune function (Pipers and De Vliegher, 2013). The mechanism behind the

immunomodulating effects of MCFA in early lactation cows is unidentified. To test the effects of MCFA on pH stabilization and inflammatory response in fresh cows, effects of MCFA supplementation should be evaluated in diets differing in starch content (high vs. low). I propose supplementing MCFA at 0.25% of dietary DM for 21 days in the post-partum diets. Blood metabolite concentrations and inflammatory markers will be evaluated. Ruminally cannulated cows can be used for continuous measurements of rumen pH. Based on the results observed in Chapter 2, I speculate MCFA will be able to control the daily variations in rumen pH and increase the minimum rumen pH of transition cows. This may prevent incidences of SARA, DMI depression and increase the health of the animal. I also speculate the metabolic energy status of postpartum dairy cows would be improved due to a more consistent energy supply to the host (Oba and Allen, 2000) and reduction in the severity in inflammation.

3.3.3 Influence of MCFA on rumen pH regulation

In Chapter 2, we observed an increase in minimum rumen pH and a decrease in the daily range of rumen pH. In addition, we observed changes in ruminal microbiota relative abundance; concluding that MCFA fed at a low dose impacts rumen fermentation. However, the mode of action in regulating ruminal pH when supplementing MCFA has not been specified. Rumen pH is a balance between VFA production and the removal of acid through absorption, neutralization and digesta passage (Allen, 1997). In Chapter 2, we observed no changes in total VFA concentrations or VFA profile when supplementing MCFA, however we cannot assume the rate of VFA production and absorption are equal.

The rate of VFA production is dependent upon substrate composition and availability, rate of depolymerization, and microbial species present (Dijkstra, 1994). In chapter 2, we observed changes in microbial species relative abundance which may have influenced VFA

production. A decrease in microbial cell yield will decrease the production of VFA (Allen, 1997); a decrease in rumen acid production will increase rumen pH. One theory for observing increased minimum rumen pH is VFA production decreased in animal supplemented with MCFA.

The rate of VFA absorption is dependent upon chain length, pH and concentration gradients (Dijkstra, 1994). When rumen pH increases, few VFA are in the associated form, and absorption rate of acid via diffusion is reduced (Allen, 1997). If MCFA are absorbed and metabolized by rumen epithelium an increase of cellular energy would occur; the active transport capacity of rumen epithelium may be increased. In addition, calves` supplemented with short- and medium-chain fatty acids, cytoplasmic protrusion on the papillary tip increased, suggesting greater absorbent surface area on the ruminal mucosa (Ragionieri et al., 2016). Thus, our second theory for observing increased minimum rumen pH is VFA absorption increased in animals supplemented with MCFA.

Bicarbonate from saliva or rumen epithelium work together to neutralize acids in the rumen. It is not likely that MCFA supplementation influence the buffering capacity of saliva to regulate rumen pH. As experimental diets were the same in forage fiber content as well as particle size was not manipulated; rumination time of animals, theoretically, would not have been altered by the MCFA supplemented diet.

The lack of treatment effect on VFA concentrations may be due to sampling and analysis methods used in Chapter 2. Volatile fatty acid production and concentration is highest approximately 4-hours post feeding (Pless et al., 2018). In Chapter 2, eight time points were chosen to collect rumen fluid. Samples were collected every 9-hours and combined as one to represent every 3-hours in a 24-hour period (e.g., 3, 6, 9, 12, 15, 18, 21 and 24 hours post

feeding). Rumen fluid samples collected after feeding, when the treatment effects are expected, were not analyzed separately, and this may have contributed to not detecting significant treatment effect on VFA concentrations. This speculation is consistent with rumen pH data, in which we detected a tendency of treatment effect on minimum rumen pH, when VFA production and concentration would be highest, while we failed to detect treatment effects on overall mean rumen pH.

To test effects of MCFA on rumen pH regulation, I propose a preliminary study using Ussing chambers to determine if MCFA can diffuse across the rumen epithelium. In addition, ruminally cannulated animals could be used to collect rumen tissue and rumen digesta; along with continuous measurements of rumen pH and calculate the rate of VFA production and absorption. Rumen tissue can be used to evaluate morphology and genes coding for epithelial growth, VFA transport and VFA metabolism. Rumen digesta can be used to evaluate microbial diversity and abundance.

I speculate that MCFA may diffuse across rumen epithelia and are metabolized as an energy source by epithelial cells. Surface area of rumen mucosa may increase in lactating cows when supplementing MCFA, increasing pH regulating capacity of rumen epithelium. However, no study has measured morphological changes to rumen epithelium when feeding MCFA. In addition, recent research has indicated that higher populations of epimural bacteria are associated with increased rumen pH, suggesting that epimural bacteria may play a stimulatory role in rumen epithelium absorption of VFA (Chen et al., 2012; Petri et al., 2020). Based changes in rumen microbiota diversity and abundance, observed in Chapter 2, I speculate that epimural bacteria populations are increased when supplementing MCFA, increasing the absorption rate of VFA and aid in the regulation of rumen pH.

3.3.4 Low dose of MCFA on methanogenesis

Supplementing MCFA *in vitro* has been shown to decrease methane production (Dohme et al., 2001; Machmuller et al., 2001). Medium-chain fatty acids supplementation rates were greater than 3% of dietary DM, which decreased NDF digestibility and VFA production. In Chapter 2, we observed no difference in NDF digestibility and VFA concentrations when supplementing MCFA at 0.063% of dietary DM. However, no data exist on methane production when MCFA are supplemented at a low dose of dietary DM.

In Chapter 2, we observed changes in relative abundance of methane producing species when supplementing a low dose of MCFA. Previous *in vitro* studies do not identify archaea species diversity and abundance. In addition, the increase in minimum rumen pH observed in Chapter 2 may increase the growth and activity of methanogens. To evaluate low dose supplementation of MCFA on methane emission, a rumen simulation technique would be used for total collections of methane emissions along with a microbial analysis of archaeal species. I speculate that supplementing a low dose of MCFA *in vitro*, would increase methane emissions and alter methanogen species abundance to enhance methane production.

3.4 Conclusion

In conclusion, supplementing MCFA at 0.063% of dietary DM to mid-lactation cows did not affect DMI, nutrient digestibility and milk yield. Supplementing a low dose of MCFA increased minimum rumen pH and decreased the daily range of ruminal pH which may be beneficial to ruminal energy absorption and health of the animal. Rumen microbiota relative abundance is affected by MCFA supplementation, most notably in methanogen species, suggesting that even at a low dose, MCFA still alter rumen fermentation and rumen microbiota.

Further research would be beneficial to expand the knowledge of low dose MCFA supplementation on rumen pH and rumen microbiota.

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