RUMINAL CHARACTERIZATION OF LIPASE-PRODUCING BACTERIA AND IMMUNOGENIC INHIBITION OF RUMINAL LIPASE ACTIVITY: EFFECTS ON RUMEN MICROBIAL ECOLOGY AND BEEF QUALITY

A Dissertation

by

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ABSTRACT

Ruminants saturate up to 80% of dietary unsaturated fatty acids through the microbial processes of lipolysis and biohydrogenation, promoting the accumulation of saturated fatty acids in their meat and milk. Studies were conducted to characterize and define contributors to the ruminal saturation of fatty acids and determine a method to minimize this process. The lipase-producing bacteria *Anaerovibrio lipolyticus* 5s, *Butyrivibrio fibrisolvens* 49, *Propionibacterium avidum* and *Propionibacterium acnes* were characterized in the presence and absence of glucose, and glycerol. Accumulations of free fatty acids (FFA) were compared between ruminal mixed cultures and each individual pure culture; these ruminal lipase-producing bacteria behaved differently from ruminal mixed cultures. A subsequent study characterized heat-treated ruminal mixed cultures that was selective for *Clostridium* in the presence and absence of glycerol. Ruminal mixed cultures behaved similarly to heat-treated cultures in the presence of glycerol, suggesting that *Clostridium* species may contribute appreciably to ruminal lipolysis. In an attempt to reduce ruminal lipolytic activity, antibodies were generated against *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum*, and *P. acnes*. Each antibody was tested against each bacterium in pure culture. An antibody generated against a purified *Pseudomonas* lipase also was tested against each pure culture to determine if an antibody against a purified lipase would be more effective than antibodies raised against whole cells. All five antibodies were effective at reducing lipolytic activity across all pure cultures of bacteria, with the anti-lipase antibody showing the greatest reduction. A

subsequent study examined the four, whole cell antibodies against ruminal mixed cultures and demonstrated that whole cell antibodies also were effective at reducing lipolytic activity of ruminal mixed cultures. Uniquely, *B. fibrisolvens* participates in both lipolysis and biohydrogenation. Thus, anti-*B. fibrisolvens* H17C antibody also was examined to determine if it would be effective at reducing biohydrogenation against *B. fibrisolvens* H17C in pure culture and ruminal mixed cultures. The anti-*B. fibrisolvens* H17C antibody depressed biohydrogenation in pure and mixed cultures. These studies demonstrated that ruminal bacteria responsible for freeing and hydrogenating ruminal unsaturated fatty acids can be immunologically inhibited *in vitro*.

DEDICATION

To my parents, Tyler and Marcy Edwards for their much needed support and love which carried me over ever obstacle no matter how impossible and to Derek Steinbring who's encouragement and love enabled me to persevere.

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INTRODUCTION AND LITERATURE REVIEW

Health Effects of Dietary Lipids

Dietary fats and oils represent a significant percentage of the human daily caloric intake in the United States, comprising >33% of total calories (Ursin, 2003). Current recommendations limit total fat intake to <35% of daily calories, 7% of that comprised of saturated fatty acids and the remainder from monounsaturated and polyunsaturated fatty acids (PUFA) (Lichtenstein et al., 2006). From its peak in 1976, red meat consumption dropped 23 pounds per person by 2005; declines in beef consumption was largely attributed to this decline in meat consumption, dropping 22% between 1970 and 2005 (Wells and Buzby, 2008). The intake of fat from red meat has been a public health concern since it was recommended that dietary cholesterol, saturated fat, and total fat be reduced for the prevention of cardiovascular disease in the 1950s (Lichtenstein et al., 2006).

Diets that contain a high content of saturated fats have been associated with a number of negative health effects, such as increased serum cholesterol levels and risk of coronary heart disease (Wahrburg, 2004). Endothelial dysfunction is an early and critical event in pathogenesis of atherosclerosis and cardiovascular disease (Ross, 1993). Endothelial dysfunction has typically been shown to precede the development of atherosclerotic plaques in coronary arteries (Mano et al., 1996; Ross, 1999). The vascular endothelium releases nitric oxide, synthesized from L-arginine, which is

responsible for maintaining vasodilation and cardiovascular homeostasis (Kubes et al., 1991; Boger et al., 1997). Omega-3 fatty acids have been found to inhibit the age-related reduction in L-arginine (Eid et al., 2006).

Saturated fatty acids, such as palmitic (C16:0), lauric (C12:0), and myristic (C14:0) work to influence cholesterol by reducing low density lipoproteins receptor activity, which decreases the cellular uptake of low-density lipoproteins uptake (Dietschy et al., 1993). High-density lipoproteins (HDL) are also affected by saturated fats. These lipoproteins are involved in lipid transport and are the predominant dietary mechanism for regulating low density lipoproteins (LDL) metabolism (Wijendran and Hayes, 2004). Dietary cholesterol comes exclusively from animals sources and is an important component of cell membranes and a precursor of bile acids, steroid hormones, and vitamin D (Lecerf and de Lorgeril, 2011). The effects that saturated fats have on cholesterol are a major factor that leads to the ultimate development of health concerns. The replacement of *trans*-fatty acids with unsaturated fatty acids has been shown to be the most effective measure for improving blood lipid profiles (Mensink et al., 2003).

The quality of dietary lipids for human consumption also has been shown to be of importance for the development of insulin resistance and related metabolic syndromes such as blood lipid disorders, hypertension, propensity for thrombus formation, abdominal obesity and Type II diabetes mellitus (DeFronzo and Ferrannini, 1991). Vessby et al. (2001) showed that decreasing saturated fatty acids and increasing monounsaturated fatty acids improved insulin sensitivity; however, it did not appear to have an effect on insulin secretions in humans. A similar study by Summers et al. (2002)

showed that insulin sensitivity and plasma LDL cholesterol concentrations improved, as well as a decrease in the abdominal subcutaneous fat area, with a diet rich in PUFA when compared to a diet high in saturated fatty acids. The health benefits associated with PUFA are brought about by their influence on metabolic changes by inducing transcription of genes encoding proteins involved in lipid oxidation and suppressing the expression of genes encoding proteins involved with fatty acid synthase and acetyl-CoA carboxylase (Jump and Clarke, 1999; Xu et al., 1999; Clarke, 2001).

The n-6 and n-3 PUFA are essential fatty acids that cannot be synthesized by mammals and therefore must be obtained from dietary sources (Sessler and Ntambi, 1998). Linoleic (18:2n-6) and α-linolenic (18:3n-3) acid are essential fatty acids which are metabolized to provide eicosanoids, substances that possess hormone-like activity which are responsible for the regulation of many body functions (Akoh and Min, 2008). Alternatively, human diets that contain a high ratio of n-6/n-3 fats are at risk for low grade chronic inflammation which can contribute to Alzheimer's disease, cancer, coronary heart disease, metabolic syndrome, obesity, type II diabetes, osteoporosis, and dry eye syndrome (Morris, 2008b). By increasing the intake of n-3 fats it will aid in decreasing the risk of developing a chronic disease. The recommended ratio intake should be between 4:1 and 10:1 (Medicine, 2002; Gebauer et al., 2006).

Dietary Sources of Linoleic and Linolenic Acid

Polyunsaturated fats are an important component in the dietary needs of animals, especially those of the n-6 and n-3 family of fatty acids. Linoleic acid is a primary and

essential fatty acid that represents the basis of the n-6 family. Linoleic acid is considered an essential fatty acid due to the inability of animals to manufacture it, and it must be supplemented through the diet.

Epidemiological evidence suggests that linoleic acid lowers the risk of coronary heart disease (Hu et al., 1999). Linoleic acid is one of the most potent fatty acids in reducing plasma total cholesterol and low density lipoproteins (Hayes, 2000; Mensink et al., 2003). Woollett et al. (1992) fed hamsters hydrogenated coconut oil and linoleic-rich safflower oil. When the safflower oil replaced the saturated fatty acid-rich coconut oil, LDL cholesterol production rate decreased 155-200%, resulting in a 75% net decline in LDL cholesterol.

Linolenic acid is the second essential fatty acid and can be elongated and desaturated to form 22-carbon fatty acids in the n-3 family (Wijendran and Hayes, 2004). Seed oils such as flaxseed or linseed oil are one of the richest sources of linolenic acid, and can also be obtained from the thylakoid membranes of green leaves from broad leaf plants, therefore providing herbivores with a dietary source of linolenic acid.

Oleic, linoleic and linolenic acid are among the most common types of unsaturated fatty acids. They are classified as unsaturated fatty acids by the existence of double bonds in their carbon chain structure. These double bonds are in a *cis* orientation. In this configuration the hydrogen atoms of the two carbons that are double-bonded are on the same side as the double bond. This results in a bend or kink in the acyl chain structure due to the strain that the associating hydrogen atoms create from their close proximity. However, unsaturated fatty acids can exist in a *trans* orientation, when the

hydrogen atoms are located on opposite sides of the double bond, which results in a straight-chain configuration. The *trans* orientation is only created when the *cis* double bond is isomerized. Microorganisms found in the rumen are responsible for isomerizing the *cis* configuration to *trans* orientation.

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA), (Figure 1.1) is an intermediate of ruminal metabolism of dietary linoleic and α-linolenic acid. Thus, CLA is found in ruminant products and not in non-ruminants or in vegetable oils

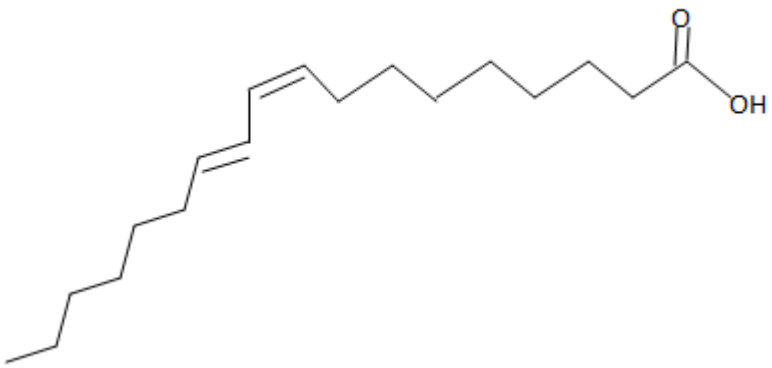

Figure 1.1 *Cis*-9, *trans*-11 conjugated linoleic acid (CLA), a bacterial isomerization product of linoleic and linolenic acid. Found naturally in ruminal products as a result of incomplete biohydrogenation. The double bonds are located at the 9^{th} and 11^{th} carbon from the carboxyl end of the fatty acid. This particular CLA is associated with several beneficial health effects.

(Chin et al., 1992; Givens and Shingfield, 2004). Ruminant fats are, therefore, one of the richest natural sources of CLA (Chin et al., 1992). Conjugated linoleic acid is an unsaturated fatty acid that contains two double bonds. These double bonds can be present as *trans/trans, trans/cis*, or *cis/cis* in structure; CLA can therefore exist in the form of a *trans*-fatty acid.

Conjugate linoleic acid, in particular the *cis*-9, trans-11 isomer, has been implicated to contribute to cancer prevention, improved immune response, altered protein and energy metabolism, and decreased atherosclerosis (Whigham et al., 2000; Pariza, 2004; Palmquist et al., 2005). Conjugated linoleic acid originates from ruminal isomerization, a process that requires lipolysis (Bauman et al., 2000). It was originally indicated that CLA was derived primarily from linoleic acid in the rumen (Kelly et al., 1998a), but Bessa et al. (2000) later showed an alternative pathway that derived CLA from α-linolenic acid (Figure 1.2). Choi and Song (2005) supplemented oils rich in either linoleic or α-linolenic acid into mixed ruminal cultures *in vitro* and found that the production of the CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 was slightly higher in cultures with α-linolenic acid than in those cultured with linoleic acid. Similarly, Wang et al. (2002) reported CLA production was greater from sources high in α-linolenic acid than from sources high in linoleic acid.

Cattle fed hay or pasture-based diets, which are relatively rich in α-linolenic acid, have elevated CLA isomers in their tissues (Beam et al., 2000; Daley et al., 2010). Archibeque et al. (2005) reported the fatty acid composition of adipose tissues of steers fed corn- or flaxseed-based finishing diets, there was a greater concentration of CLA in

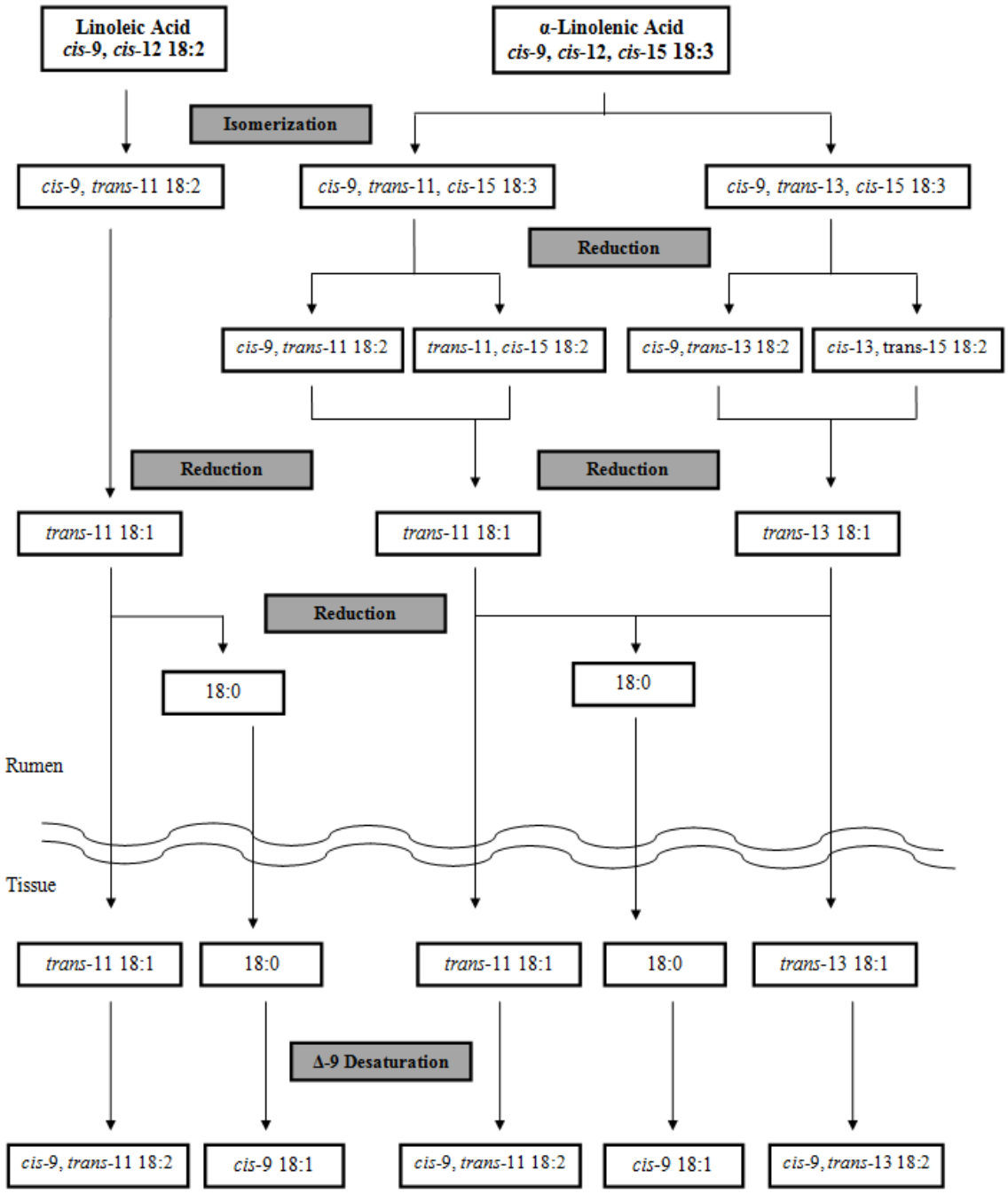

Figure 1.2 Ruminal biohydrogenation of linoleic and α-linolenic acid and endogenous tissue delta-9-desaturase metabolism (Destaillats et al., 2005). Both linoleic and α-linolenic biohydrogenation pathways produce the health benefitting isomer *cis*-9, *trans*-11 CLA.

adipose tissues of flaxseed-fed cattle than in corn-fed cattle. Several studies have shown that including grass in the diet of dairy cows has been correlated with an increase in CLA concentration in their milk (Stanton et al., 1997; Kelly et al., 1998b; Lawless et al., 1998).

Fatty Acid Composition and Meat Quality

Previous studies suggested that the fatty acid composition of ruminant meats can have an influence on meat flavor, along with other important quality attributes, and this is highly influenced by the diet given to the animal as reviewed by Wood et al. (1999). Greater

concentrations of oleic acid are positively correlated with overall palatability of meat (Westerling and Hedrick, 1979). Varying melting points associated with individual fatty acids affects oiliness and firmness of adipose tissue in meat (Wood et al., 2008). The normal fatty acid composition of meat has a melting point between 25°C and 50°C, with saturated fatty acids melting at higher temperatures and MUFA and PUFA at lower temperatures (Wood, 1984).

Ruminal diets rich in grains have increased levels of unsaturated fatty acids in adipose tissue than cattle fed predominantly forage (Kemp et al., 1981; Enser et al., 1998). Daniel et al. (2004) showed that feeding sheep concentrate-based diets increases the oleic acid content of their tissues. In addition, a study using Limousin-cross steers compared forage versus grain feeding on carcass composition and palatability attributes of beef (Mandell et al., 1998). This study showed that the palatability attributes of ribeye

roasts and ground beef were generally unaffected ($P > 0.10$) but had a tendency for slightly less beef flavor and more off flavor in forage-fed versus grain-fed beef. Higher ($P < 0.01$) concentrations of linolenic acid and lower ($P < 0.10$) concentrations of oleic acid in forage-fed beef may have been responsible for diet differences in flavor (Mandell et al., 1998). Oleic acid is the main fatty acid in the intramuscular fat of cattle and sheep, and has been positively correlated with cooked beef fat flavor (Larick and Turner, 1990).

When α-linolenic acid levels are raised in lamb and beef in response to grass feeding, the intensity of the flavors increases in comparison with grain-fed animals, which deposit relatively more oleic and linoleic acid (Wood et al., 1999). Lipid oxidation products have been found at higher levels in the aroma extract of steaks with increased PUFA content, following cooking (Elmore et al., 1999). Grass-fed beef contains higher concentrations of di-terpenoids, derivatives of chlorophyll called phyt-1-ene and phyt-2-ene, which change the overall flavor and aroma of the cooked product (Elmore et al., 2006).

Digestion and Absorption of Lipids in Ruminal Animals

Dietary fatty acids are metabolized as fuel for oxidative phosphorylation or during a time of an energy surplus, they are stored as triacylglycerols (TAG), or rapidly incorporated into plasma phospholipids, high-density lipoprotein particles or cell membranes (Rennison and Van Wagoner, 2009). The initial step in lipid metabolism is the hydrolysis of the ester linkages linking fatty acids to their respected backbone of TAG, phospholipids, and glycolipids. Hydrolysis and also biohydrogenation processes,

as shown in Figure 1.3, occurs in the rumen as a result of microbial metabolic activity (Shorland et al., 1955; Viviani, 1970).

Microorganisms are responsible for reducing the double bond found in unsaturated fatty acids, leaving the carbons free to attach to hydrogen molecules; this effectively transforms unsaturated fatty acids into saturated fatty acids (Doreau and Chilliard, 1997). Biohydrogenation is a detoxification process, necessary for bacteria to escape from the bacteriostatic effects of unsaturated fatty acids (Maia et al., 2010). In order for biohydrogenation to occur, fatty acids must first be hydrolyzed from their TAG precursors (Harfoot and Hazlewood, 1997), a process known as lipolysis. The initial step to biohydrogenation, the isomerization reaction, requires a free carboxyl group (Kepler et al., 1970), thus establishing lipolysis as a prerequisite for biohydrogenation. Lactating dairy cows, on average, consume around 300 g of linoleic acid daily. Of this, about 40 g remains unsaturated and reaches the small intestine intact; major PUFA that do escape ruminal biohydrogenation and reach the small intestine, linoleic acid is more rapidly taken up and reaches much higher levels in meat tissues than α-linolenic acid. Synthesis of long chain PUFA from these precursors leads to enrichment in phospholipids but the available sites for incorporation are quickly filled and long term feeding of grass to cattle does not increase levels of these unsaturated fatty acids (Warren et al., 2008; Wood et al., 2008).

One property of hydrogenating ruminal bacteria is the ability to biohydrogenate linoleic acid and α-linolenic acid to *trans*-vaccenic acid and stearic acid (Harfoot and Hazlewood, 1997). In ruminants, most hydrolysis of complex lipids occurs in the rumen

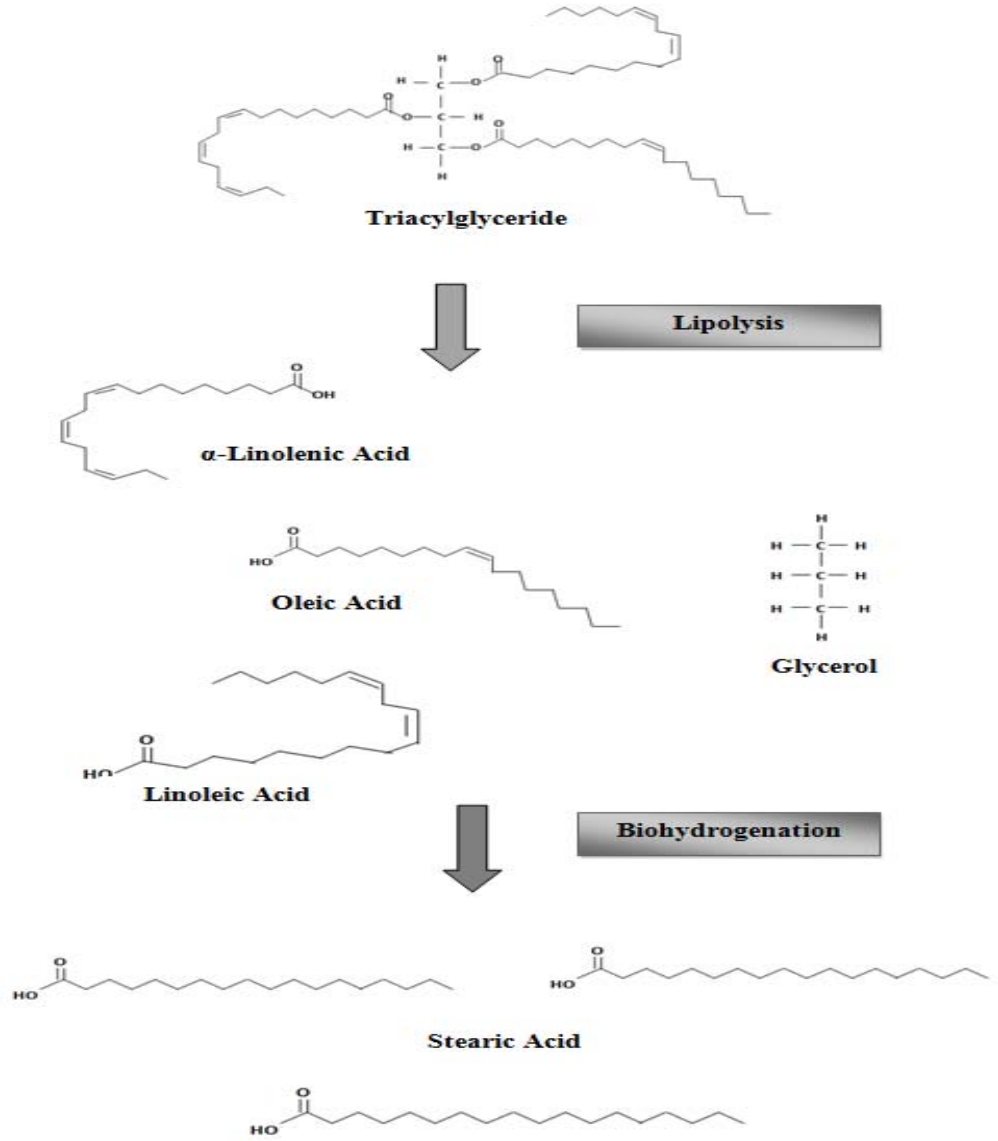

Figure 1.3 The breakdown and saturation of a triacylglyceride through the microbial mediated processes of lipolysis and biohydrogenation. Lipolysis is a pre-request for biohydrogenation because of the free carboxyl group biohydrogenation requires. Bacteria target the glycerol backbone of a triacylglyceride for energy production. The bacteria produce a lipase which frees the fatty acids from the glycerol backbone. The lipase frees the fatty acids at the n-1 and n-3 fatty acids first and then subsequently frees the fatty acid at the n-2 position. Once free, the fatty acids are then available for microbial saturation. The figure shows three 18-carbon unsaturated fatty acids being saturated to 18 carbon stearic acid.

by action of microbial lipases (Harfoot, 1978). However, some dietary esterified lipids may escape the rumen and be digested and absorbed in the small intestine. These esterified lipids are in the predominant form of microbial phospholipids and phospholipids from bile and sloughed intestinal cells (Sejrsen et al., 2008). Choi and Song (2005) examined the effect of 18-carbon PUFA on direct incorporation into rumen bacteria by adding 60 mg of linoleic acid or α-linolenic acid into a rumen fluid, mixed culture solution. The amount of fatty acids incorporated into the bacteria following a 12-h incubation were 1.20 mg and 0.43 mg/30 mL rumen fluid for linoleic and α-linolenic acid, respectively (Choi and Song, 2005).

Beam et al. (2000) examined the amount and source of fat on rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. Results showed that the rate of lipolysis of soybean oil declined from 44%/h to less than 30%/h as the percentage of soybean oil in the culture substrate increased from 2 to 10% and the rate of biohydrogenation of linoleic acid was 14.3%/h, but declined 1.2%/h for each percentage unit increase in linoleic acid added to the substrate. Based on these results, Beam et al. (2000) demonstrated that a diet high in linoleic acid would possibly reduce biohydrogenation and increase the post-ruminal flow of unsaturated fatty acids. In addition to this, Edwards et al. (2013) recently provided evidence *in vitro* that flaxseed oil, rich in α-linolenic acid, was more readily hydrolyzed than corn oil rich in linoleic acid.

The higher content of unsaturated fatty acids in tissues of corn fed cattle was originally considered to be a product of the low pH in the rumen caused by the corn,

resulting in reduced bacterial activity. Van Nevel et al. (1996) showed that lipolysis was decreased at pH $\leq$ 6.0 with biohydrogenation only showing marginal inhibition at a lower pH. However, Van Nevel et al. (1996) concluded that since lipolysis and biohydrogenation were not inhibited to a greater degree at a low pH, other factors must be involved in the decrease of both lipolysis and biohydrogenation in the rumen of animals fed highly concentrated diets. Thus, cattle fed a corn-based diets having adipose tissue high in unsaturated fatty acids is likely a combination of the protective effects of linoleic acid against lipolysis in the rumen and its rapid assimilation into tissue.

Fatty acids that are stored in tissue can come from two sources: either through dietary lipids or by synthesis from other carbon sources. *De novo* fatty acid synthesis occurs by acyl carrier protein (ACP) synthase enzymes which construct fatty acid carbon chains using a repeated multi-reaction process. The reaction starts with a *trans* thio-esterification of the acyl primer substrate from the acyl carrier protein to the enzyme. Subsequently, the donor substrate malonyl-ACP is decarboxylated to form a carbon ion intermediate, which then attacks the first carbon of the primer substrate giving rise to an elongated acyl chain (Brady and Gurin, 1952; von Wettstein-Knowles et al., 2006). These reactions are continually repeated adding two carbons to the chain each time.

The fatty acid synthase enzyme is unable to produce a fatty acid carbon chain longer than palmitic acid. However, palmitic acid only comprises 20 to 25% of the fatty acid composition of beef (Sumida et al., 1972; Westerling and Hedrick, 1979; Manner et al., 1984). Thus, elongation enzymes add an additional two carbons creating, stearic acid. With the high melting point of stearic acid, it is too insoluble to be stored in high

concentrations (Berry, 1997). A critical step in the biosynthesis of monounsaturated fatty acids is the introduction of the first *cis* double bond at the ninth carbon by delta-9-desaturase found in the tissue of animals, giving rise to oleic acid.

Ruminal animals can also convert glucose into glycerol-3-phosphate which is used for the backbone to TAG production or acetyl-CoA, which as indicated above is a precursor for *de novo* fatty acid synthesis. Glucose can also be converted into glucose-6-phosphate which can then go to the pentose cycle to produce glycerol-3-phosphate, NADPH (needed for fatty acid synthesis), erythrose-4-phosphate (precursor to synthesize aromatic amino acids), or ribose-5-phosphate (a precursor for nucleic acid synthesis). In general, free glucose that is absorbed from the breakdown of cellulose can be used as a precursor for TAG synthesis or used for the production of energy needed for *de novo* synthesis of fatty acids (Figure 1.4).

Glucose in the Rumen

Ruminal microbes must ferment the release of sugars in order to synthesize ATP, which allows for further bacterial growth and function. Cereal grains contain 57 to 77% of dry matter as starch; other feedstuffs such as grasses and legumes contain 2 to 20% starch (Huntington et al., 2006). Carbohydrate metabolism and lipid metabolism is closely interrelated which carbohydrates can be converted to lipids, as previously described (Figure 1.4).

Similarly, lipolysis of a TAG results in a glycerol molecule and three fatty acids. The resulting glycerol molecule can then be used to produce intermediates of glycolysis.

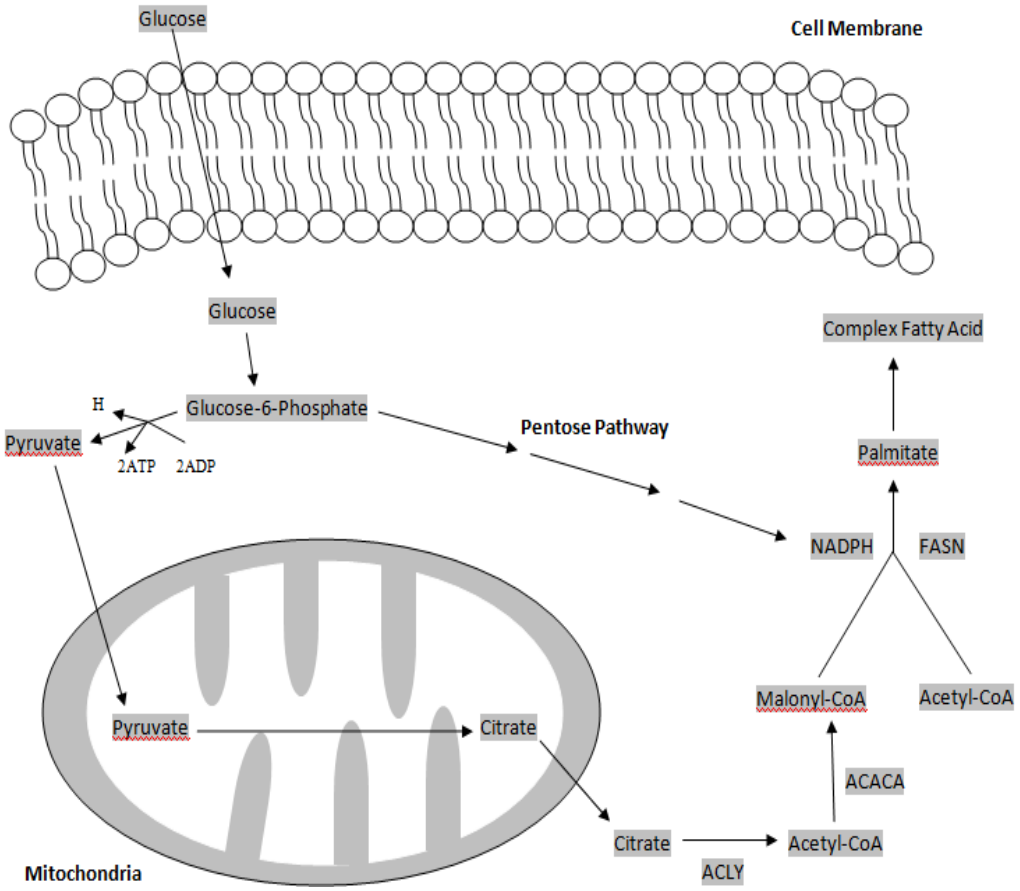

Figure 1.4 Glucose metabolism and *de novo* fatty acid synthesis (Menendez and Lupu, 2007). NADPH is used as a reaction catalyst for fatty acid synthesis which is acquired by the pentose phosphate pathway. The carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACACA) is the regulatory and rate-limiting step in *de novo* fatty acid synthesis. Acetyl-CoA carboxylase is activated by saturated long-chain fatty acids can be further modified by elongases or desaturases.

For almost all living cells, glucose is the primary energy source utilized and as such glucose content in feedstuff can be a major player in the regulation of lipolysis and biohydrogenation in the rumen. A high concentration of fermentable starch in the diet is associated with increased production of organic acids, increased production of microbial protein, decreased fiber digestion, decreased ammonia concentrations, and decreased acetate: propionate ratio (Poore et al., 1993; Martin et al., 1999; Philippeau et al., 1999). Propionate is absorbed through the rumen wall and is synthesized into glucose or used to make fatty acids depending on the animal's needs. Ruminal fermentation of volatile fatty acids (VFAs) of acetate, propionate, and butyrate using glucose as a precursor is depicted in Figure 1.5.

An *in vivo* study Knight and Iliffe (1972) reported that glucose increased the accumulation of glycerol in adipose tissue, indicating an increase in lipolysis. Additionally, studies have shown that increasing the availability of water-soluble carbohydrate has led to conditions that have been linked to reduced biohydrogenation of dietary unsaturated fatty acids (Jenkins, 1993). Furthermore, Lee et al. (2006) showed that total fatty acid and α-linolenic concentrations were greater for the high-sugar grass silage than the control grass silage or the red clover silage. They also reported that flows of total branched- and odd-chain fatty acids were greater ($P < 0.05$) for the high-sugar grass silage diet compared to control silage. They concluded that this is possibly a result of greater microbial flow, because branched- and odd-chain fatty acids are generally associated with bacterial lipid. According to these studies, a high availability of glucose in the rumen appears to increase lipolytic activity but decrease biohydrogenation. The

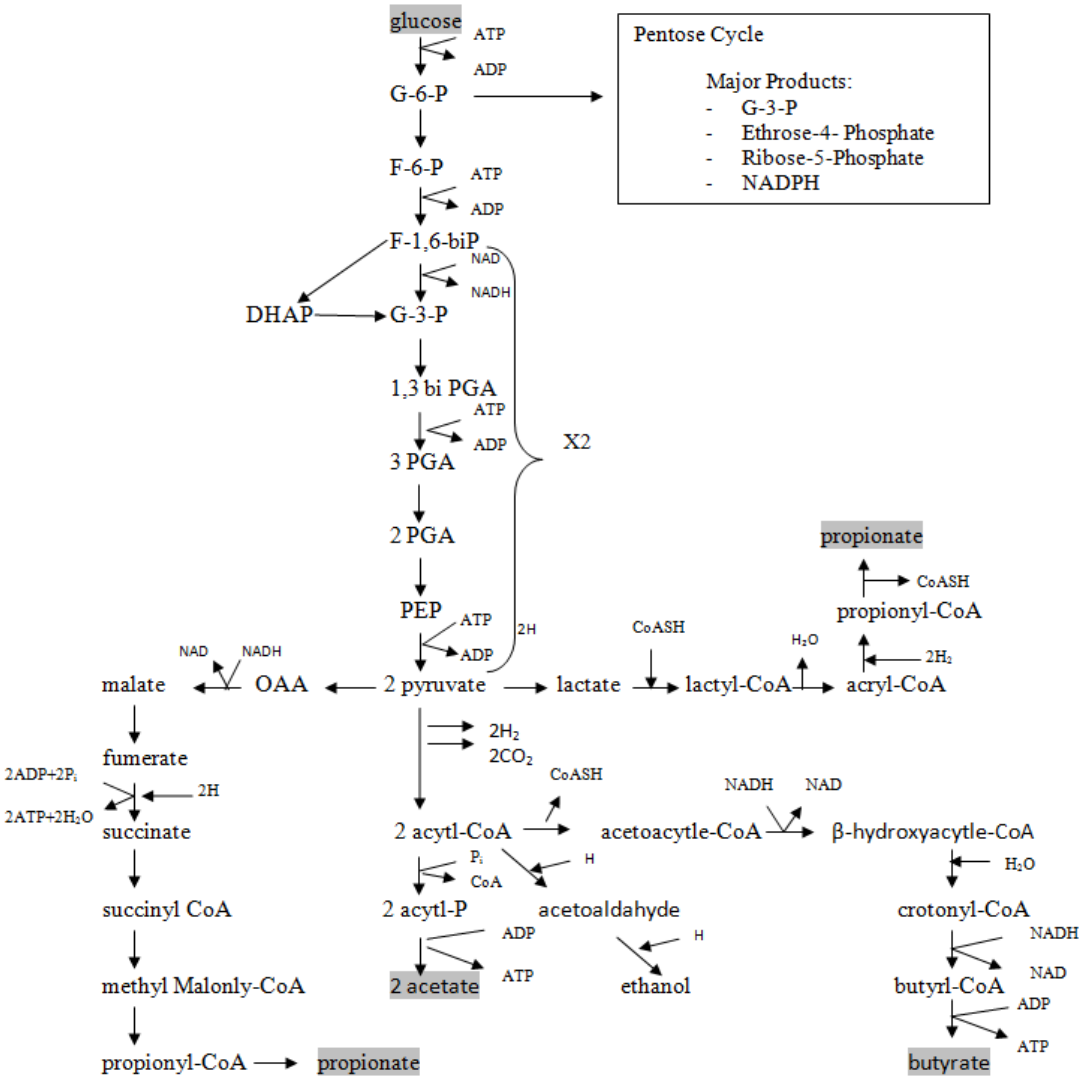

Figure 1.5 Ruminal anaerobic fermentative pathways of glucose into the volatile fatty acids (VFA) acetate, propionate and butyrate (Kim and Geoffrey, 2008). These microbial fermentative pathways produce energy and valuable products. The production of propionate from glucose serves as a hydrogen sink and also can be used as a carbon source for de novo synthesis. The fermentative pathway that glucose takes is dependent on the animal and ruminal micro biome's needs.

increase in branched chain fatty acids, however, indicates that increases in glucose may be aiding bacteria growth and possible lipase- producing bacterial growth, explaining the increase in lipolytic activity. Further research regarding increasing levels of glucose in the rumen needs to be conducted to determine the long term effects of high levels of glucose on fatty acid composition in the meat and milk of ruminal animals.

Regulation of Delta-9-desaturase Activity

Monounsaturated fatty acids, such as oleic acid, are synthesized from saturated fatty acids by delta-9-desaturase, also known as SCD. Delta-9-desaturase introduces a double bond between the 9^{th} and 10^{th} carbon of a saturated fatty acid chain (Brock et al., 2006) as demonstrated in Figure 1.2. Delta-9-desaturase thus influences both dietary lipids and *de novo* fatty acid synthesis and is a contributing factor as to the reason that oleic acid is the predominant fatty acid found in adipose tissue.

Casmir and Nambe et al.(1996) and Chung et al. (2006) provided evidence that SCD gene expression increases immediately preceding lipid filling in preadipocytes. The expression of the SCD gene is regulated by PUFA and cholesterol at the levels of transcription and mRNA stability (Ntambi, 1999). Similar to PUFA, cholesterol also represses the expression of the SCD gene and enzyme activity (Chin and Chang, 1982; Tabor et al., 1998). Furthermore, Waters et al. (2009) showed that the enhancement of tissue concentrations of CLA and n-3 PUFA concentrations in bovine muscle may be hindered by negative interactions between n-3 PUFA and SCD gene expression. In bovine preadipocytes, *trans*-10,*cis*-12 CLA has been shown to almost completely

eliminate SCD gene expression, while *cis*-9,*trans*-11 CLA only showed an effect at very high concentrations (Chung et al., 2006).

Contradictory to the previous studies, research by Archibeque et al. (2005) showed that differences in saturated fatty acids seemed to be independent of SCD enzyme activity in adipose tissues. This work suggests that the concentrations of fatty acids that reach the small intestine are a bigger factor in determining tissue fatty acid concentrations than endogenous desaturation. Data from this study also showed that 18:1*trans*-11 was not an effective substrate for SCD in the production of 18:2 *cis*-9, *trans*-11. However, studies by both Santora et al. (2000) and Corl et al. (2003) have demonstrated that increases in tissue concentrations of 18:1 *trans*-11 were associated with proportional increases in 18:2 *cis*-9, *trans*-11 in mammary tissue.

Ruminal Microorganisms

The rumen is a self-contained ecosystem where feed consumed by the ruminant is fermented to VFAs and microbial biomass. The end products serve as the source of energy and protein (Weimer, 1998). The extent to which an unsaturated fatty acid is subject to ruminal biohydrogenation is determined by the fat source, retention time, and characteristics of the microbial population (Allen, 2000). There are several different types of micro-organisms found in the rumen that are capable of lipolysis and biohydrogenation. This study focuses on two bacteria that have previously demonstrated the greatest lipolytic activity *in vitro.* These microorganisms include *P. acnes* and *P. avidum*. *Butyrivibrio fibrisolvens* 49 and *A. lipolyticus* 5s were also investigated in this

study due to previous findings indicating them as major contributors to lipolysis (Polan et al., 1964; Henderson, 1971; Henderson and Hodgkiss, 1973; Prins et al., 1975). Ruminal *Clostridium* species were also included so as to determine their contribution to overall ruminal lipolytic activity. *Clostridium* species are highly diverse in the rumen and as a whole could be contributing more to lipolytic activity as a combined genus than originally thought.

Anaerovibrio lipolyticus

Anaerovibrio lipolyticus is a flagellated, curved rod-shaped anaerobic Gram-negative bacterium that produces an extracellular lipase during exponential growth. Hobson and Mann (1961) first isolated the bacterium from the ovine rumen. Culture studies have found *A. lipolyticus* to be present in the rumen at around 10^7/mL (Prins et al., 1975) suggesting a dominant functional role with regards to ruminal lipolysis activity. The lipase produced by *A. lipolyticus* is most active at pH of 7.4 and from 20 to 22ºC, and diacylglycerides appeared to by hydrolyzed more rapidly than triacylglycerides (Henderson, 1970; Henderson, 1971; Henderson and Hodgkiss, 1973). Henderson (1971) showed that the activity of this enzyme was enhanced by $CaCl_2$ and $BaCl_2$, while $ZnCl_2$ and $HgCl_2$ worked to inhibit activity. *Anaerovibrio lipolyticus* has limited capacity for fermenting carbohydrates; only glycerol, fructose and ribose are fermented with acetic, propionic, and succinic acids being formed (Henderson, 1975). Prins et al. (1975) reported that rumen fluid was not required for the bacterium's growth

and that good growth could be obtained in a medium containing minerals, glycerol, yeast extract, and trypticase.

Henderson (1971) conducted a study in which batch cultures of *A. lipolyticus* were grown and samples removed at designated intervals, assaying the lipase in bacteria-free cultures or in re-suspended bacteria using olive oil as the lipid substrate. Henderson (1971) found that lipolytic enzyme activity began to appear in bacteria-free medium soon after exponential growth. As the bacteria entered stationary phase the enzymatic activity decreased, presumptively due to increased acidity (which subsequently was proven in pH-controlled conditions). From this experiment predictions of when maximal enzymatic activity would occur could not be determined due to variation in lag phase batch cultures. Henderson (1971) indicated that the lipase was produced extracellularly by satisfying the criteria established by Pollock (1962) for enzymes to be produced extracellularly. Henderson (1971) demonstrated that the lipase appears in the medium early in the life of the culture and that secondly the lipase activity was not associated with the bacterial cell or fragmented bacteria. Henderson and Hodgkiss (1973) provided support to the second criterion established by Pollock (1962) through an electron microscope study of cultures of *A. lipolyticus* which showed no evidence of lysed bacteria. This discounted the theory that lipase was released from *A. lipolyticus* through autolytic fragmentation.

Anaerovibrio lipolyticus is responsible for hydrolyzing galactosyl, glycerides, phospholipids, and TAG. Glycerols liberated during hydrolysis from TAG are completely metabolized to VFAs. Garton et al. (1961) found that propionic acid made up

the majority of the VFAs but it never accounted for more than 50% of the metabolism of glycerol. It is still unknown as to what happens to the remaining glycerol that is not metabolized into VFAs. Clarke and Hawke (1970) could not detect significant lipolytic activity in clarified rumen fluid and concluded that the lipase in the rumen was cell bound and not released into the surrounding media. Culture counts of *A. lipolyticus* in the rumen of sheep ranged between 0.5-1.1 x 10^7 CFU/mL which corresponds with Prins et al. (1975) finding that *A. lipolyticus* was responsible for the major part of the lipolytic activity in ruminant animals.

Butyrivibrio fibrisolvens

High numbers during isolation of *B. fibrisolvens* from ruminal fermentation has indicated that it may also be of functional importance in the rumen (Brown and Moore, 1960). *Butyrivibrio fibrisolvens* is an obligate anaerobe, curved rod, possessing a monotrichous flagellum enabling motility (Brown and Moore, 1960). *Butyrivibrio fibrisolvens* is a butyric acid forming bacteria and has been known to produce extracellular polysaccharides (Stack, 1988). However, the function of these extracellular polysaccharides is still unknown. *Butyrivibrio fibrisolvens* is universally described as a Gram-negative bacterium. However, their cell walls display characteristics that are common amongst the Gram-positive bacteria. Hespell et al. (1993) found that the cell walls contained teichoic acids, normally a constituent in a Gram-positive bacteria cell wall. The cell wall is very thin, ranging from 12 to 18 nm, which could suggest why the bacterium easily decolorized during Gram staining (Cheng and Costerton, 1977).

Butyrivibrio fibrisolvens plays a major role in lipolysis and biohydrogenation in the rumen, leaving only a small portion of PUFA to find their way into the meat and milk of ruminant animals (Maia et al., 2010). A study conducted by Maia et al. (2010) investigated the mechanisms by which PUFA affect the growth of *B. fibrisolvens* and how they are metabolized by *B. fibrisolvens*. They found linoleic and α-linolenic acid to lengthen the lag phase of *B. fibrisolvens* with linoleic acid having the greater effect. However, growth occurred only when PUFA had been converted to *trans*-vaccenic acid. They also found the major fish oil acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were not metabolized but prevented growth of *B. fibrisolvens*. *Butyrivibrio fibrisolvens* has been identified for causing to the initial reaction of biohydrogenation pathway, isomerization (Polan et al., 1964). From these results they concluded that lipolysis and biohydrogenation occur to enable *B. fibrisolvens* to survive the bacteriostatic effects of PUFA.

The first isolation of *B. fibrisolvens* was achieved from the bovine rumen (Bryant and Small, 1956). Many other strains have been isolated subsequently. Vast variability exists between the strains. Most strains of *B. fibrisolvens* are xylanolytic, while a small number have significant fibrolytic abilities (Dalrymple et al., 1999). In work done by Hespell and O'Bryan-Shah (1988), several different cultures of *B. fibrisolvens* were grown to mid- to late logarithmic growth phases prior to experimental use. The change in total culture esterase activity was monitored as a function of the culture growth stage. *Butyrivibrio fibrisolvens* 49, showed esterase activity increased with cell growth until the stationary growth phase was reached, where the activity remained stable. Cotta and

Hespell (1986) found the production of extracellular proteases to be constitutive, similar to the pattern observed for esterase activity.

Butyrivibrio fibrisolvens grows best in a rumen fluid-glucose medium containing N_2 and an absence of bicarbonate. It also grows well when rumen fluid is replaced by yeast extract and trypticase in a glucose medium incubated at 45ºC (Bryant and Small, 1956). *Butyrivibrio fibrisolvens* is capable of hydrogenating linoleic acid to octadecanoic acid but not to stearic acid. A completely anaerobic environment is required and with mixed rumen cultures more activity is obtained in an atmosphere of hydrogen versus nitrogen or helium, while carbon dioxide acts as an inhibitor (Polan et al., 1964). The presence of organisms that lack the ability to perform biohydrogenation by themselves can prevent loss of the activity of *B. fibrisolvens* due to age or dilution making biohydrogenation activity in *B. fibrisolvens* dependent on the age of the organism and the concentration of cells used in the medium (Polan et al., 1964).

Propionibacterium avidum *and* P. acnes

Propionibacterium spp. have been known to be present in high populations found within the rumen. Studies have established the effective *Propionibacterium* population in the rumen is between 10^4 and 10^6 CFU/mL of rumen fluid (Zimmer, 1999). *Propionibacterium* spp. hydrolyze only neutral lipids but not polar lipids (McInerey, 1988; Jarvis et al., 1998; Jarvis et al., 1999; Cirne et al., 2006). *Propionibacterium* can utilize the glycerol arising from the de-esterification of triglycerides and phospholipids (Jarvis and Moore, 2010).

Propionibacterium avidum is a non-spore forming Gram-positive, irregular rod shaped, facultatively anaerobic bacterium. Optimum growth for this bacterium occurs at a pH between 5.0 and 8.0. *Propionibacterium avidum* produces an extracellular lipase and proteinase (Greenman et al., 1983). Strains of *P. acnes* and *P. avidum* can be biochemically differentiated based on the ability of *P. avidum* to ferment sucrose or maltose and to characteristically hydrolyze esculin, which are properties absent in *P. acnes* (Cummins and Johnson, 1974). Cove et al. (1983) conducted a study where *P. acnes* and *P. avidum* were grown in continuous culture at 0-100% air saturation. The results show that *P. avidum* is best adapted for growth in an aerobic environment, suggesting that *P. avidum* can be identified as a facultative organism. In the same study they looked at the results of increasing the glucose concentration up to 3% in a tryptone-based medium and found that the increase in glucose caused an increase in the biomass of *P. acnes* and *P. avidum*.

Research done by Chopra and Hacker (1989) looked at the effects of tetracycline on *P. avidum* and *P. acnes*. Tetracyclines are a group of broad-spectrum antibiotics that inhibit protein synthesis. They showed that the synthesis of an extracellular lipase by *P. avidum* was twofold more sensitive to inhibition by tetracycline than total cellular and extracellular protein synthesis. Other studies have been done with tetracycline and its effects on *E. coli*. Several studies reported that tetracycline inhibited proteins that were to be exported from the cell rather than cytoplasmic proteins (Hirashima et al., 1973; Piovant et al., 1978; Chopra and Linton, 1987; Schifferli and Beachey, 1988). Chopra

and Hacker (1989) concluded that tetracycline may not partition so readily into the cytoplasmic membrane of Gram-positive organisms as it does with *E. coli*.

Chopra and Hacker (1989) believed that the difference in tetracycline's ability to enter through the cell membrane was related to membrane phospholipid composition and that tetracycline inhibition abilities are influenced by the type of phospholipids it is exposed to. Results done from these studies have shown that tetracycline has the ability to inhibit the extracellular lipase produced by *P. acnes* and *P. avidum*. Evidence obtained in this study demonstrated that *P. acnes* and *P. avidum* are contributors to lipolysis of dietary fats in the rumen.

A study by Holland et al. (1979) investigated batch cultures incorporating glucose, fructose, glycerol, or arginine in the medium. The results showed that the concentrations as well as the type of carbon source used had effects on extracellular enzyme production by both *P. acnes* and *P. avidum. Propionibacterium avidum* showed increased cell yields corresponding to increased concentrations of fructose until 0.5% (w/v) was reached in the medium. From this point, the yield remained constant despite further increases of fructose. Similar results were seen with arginine except the yields obtained at any given concentration of arginine were less in each case than the corresponding yields when *P. avidum* was grown in fructose. The extracellular enzyme of *P. avidum* displayed the same pattern when grown in both fructose and arginine with activity increasing until the 0.25% concentration of those carbon sources was reached. From there, the activity remained constant even with increasing amounts of these carbon sources. Glucose appeared to suppress lipase activity of *P. avidum* while glycerol had

little effect. Further results from this study showed that the carbon source had little effect on the lipase activity of *P. acnes*.

Much like *P. avidum, P. acnes* is a Gram-positive, irregularly rod-shaped, facultative anaerobic bacterium. *Propionibacterium acnes* produces a wide range of extracellular enzymes including a lipase. Optimal growth for *P. acnes* occurs between pH 4.5 to 7.5 (Greenman et al., 1983). The bacterial lipase of *P. acnes* shares very little amino acid homology to other bacteria and as a result there is limited similarity to other ruminal lipases (Jaeger et al., 1994). The lipase nucleotide sequence (*gehA*) was found to be expressed in *P. acnes* as a 33kDa polypeptide (Lee and Iandolo, 1986). Miskin et al. (1997) confirmed this by assaying crude culture supernatant fluid for lipase activity. The extracellular lipase was produced from cells in the post-exponential phase of growth and the lipase concentration remained constant at 0.17 U/mL after 60 h incubation. Ingham et al. (1981) discovered that the lipase has the ability to hydrolyze trilaurin, triolein, trimyristin, and tripalmitin; however, the lipase did not exhibit phospholipase activity. The reaction products from the hydrolysis of triolein by *P. acnes* were analyzed and the results suggested that the enzyme did not demonstrate a positional specificity for the *sn*-1 position of the triacylglycerol.

Propionibacterium acnes displays lipase, hyaluronase, lyase, phosphatase, and proteinase activity (Greenman et al., 1983). The high activity of *P. acnes* makes it a major contributor of lipolysis and biohydrogenation in the rumen. Wallace et al. (2006) suggests that *P. acnes* may be responsible for the formation of *trans*-10, *cis*-12-18, but it did not metabolize conjugated linoleic acid isomers any further. Magasanik (1961) found

that an excess of carbon could inhibit the formation of extracellular products by the mechanism known as catabolite repression. Catabolite repression is where a freely available carbon energy source represses the synthesis of the redundant enzymes. Holland et al. (1979) conducted a study using different carbon energy sources and measured their effects through the lipase activity of the extracellular enzyme of different strains of *Propionibacterium*. The results for extracellular lipase activity from the study showed that glucose and glycerol suppressed activity of *P. acnes* strain P37; however, they had little effect on the *P. acnes* strain PF276. Glucose also appeared to suppress the lipase activity of *P. avidum*.

Clostridium *Species*

The genus *Clostridium* is traditionally identified as rod shaped, obligate anaerobic bacteria of which most are capable of producing endospores. *Clostridium* species constitutes a vast group of bacteria with diverse functional characteristics, some of which produce lipase. *Clostridium botulinum, C. aurantibutyricum, C. novyi, C. sporogens* (Dighe et al., 1998), *C. ghonii*, (Sneath, 1986), *C. tetanomorphum, C. tetani,* (Wilde et al., 1989) *C. ghonii*, (Sneath, 1986) *C. aerotolerans* (van Gylswyk and van der Toorn, 1987), and recently isolated *C. lundense* (Cirne et al., 2006) have been identified to produce lipase. Subsequently, a species named *Clostridium proteoclasticum* was identified as a stearate producer (Wallace et al., 2006). Moon et al. (2008) later renamed *C. proteoclasticum* as *Butyrivibrio proteoclasticus* from its 16S rRNA gene sequence. The diversity of *Clostridium* species in the rumen makes it difficult to define this genus'

role in the rumen and thus very little is known on *Clostridiums* contribution with regard to the saturation of fatty acids in the rumen.

Interfacial Activation and Lipolysis and Biohydrogenation *In Vitro*

It has been recognized that the enzymatic activity of lipases is markedly activated in environments that stabilize the hydrophobic-hydrophilic interfaces that occur at points of contact between oil and water (Paiva et al., 2000). In the absence of these interfaces, lipases have very little inter-esterification activity. Maruyama et al. (2000) conducted a study where they processed lipases in a two-phase hydrocarbon-water system that had an oil-water interface. They proceeded by taking crude lipases and adding them to a buffer and a small volume of aliphatic hydrocarbon, that were then mixed and lyophilized to remove the aqueous and oil phases. From this they were able to compare interfacially processed lipase to the crude lipase; it was demonstrated that the interfacially-processed lipase had significant inter-esterification activity as compared to the crude native lipases. This provides further evidence that the lipase is not cell bound. By the lipase not being surrounded and protected by a cell wall it should have ample exposure to any lipids present resulting in lipolysis. With insufficient interfacial activation, the lipase may not gain exposure to the present lipid, which would cause a reduction in lipolytic activity.

As reviewed by Lourenço et al. (2010), the ability of ruminal microbes to hydrolyze TAG was reported more than 50 years ago (Garton et al., 1958). Since then, numerous studies have been conducted to characterize the biological and physical factors affecting ruminal lipolysis by mixed or pure populations of ruminal bacteria *in vitro*.

Singh and Hawke (1979) examine lipolysis and biohydrogenation of monogalactosyldiglyceride *in vitro*. They showed that only the particulate fraction of rumen fluid gave patterns of hydrogenation similar to that obtained with rumen fluid. Bacteria in free suspension had little ability to hydrogenate trienoic fatty acids beyond the dienoic stage and neither protozoa nor the cell-free supernatant gave significant biohydrogenation of trienoic fatty acids. Furthermore, Hawke and Silcock (1970) have showed that more than 50% of ruminal lipase activity was contained with the particulate fraction of freshly collected ruminal fluid. Thus, these studies may not have been able to detect substantial lipolytic activity in the rumen fluid without a particulate fraction because there was inadequate interfacial activation to cause sufficient lipase activity. Studies using rumen contents as incubation materials likely provided a solid support that served to stabilize the hydrophobic-hydrophilic interface, a phenomenon referred to as interfacial activation. A major limitation, however, of studies conducted with particulate matter and digesta is that these materials are not homogenous in size or microbial composition which can then lead to considerable variation and experimental error during their use in *in vitro* incubations.

Factors that Effect Fatty Acid Composition in Ruminants

Fatty Acid Composition as a Result of Time on Feed

A study by Duckett (1993) using Angus x Hereford yearling steers assessed the effect of time on feed on the nutrient composition of beef *longissimus* muscle. Intramuscular fat content doubled ($P < 0.05$) between days 84 and 112 but did not differ

($P > 0.05$) from days 0 to 84 or from days 112 to 196. Lipid became more unsaturated as time on feed increased with MUFA content increasing by 22% (Duckett et al., 1993). Similar results were found in a study by Smith et al. (2009) which examined the amount of time on feed and found that both the amount of marbling and the concentration of MUFA increased with time on feed in grain-fed and pasture-fed cattle, but much more extensively in grain-fed cattle. Conversely, Sami et al. (2004) showed that time on feed had only a small effect on the fatty acid composition compared with feeding intensity. Results showed that intensive feeding decreased the ratio of polyunsaturated: saturated fatty acids when slaughtered at 100 and 138 days to 0.17 and 0.14 ($P < 0.05$) in comparison with 0.20 during an extensive feeding program with no change at different days to slaughter.

Fatty Acid Composition as a Result of Genetics and Breed

Japanese Black Wagyu steers derived from 34 sires were used to investigate genetic effects on the fatty acid composition of carcass fat (Oka et al., 2002). Results from this study suggested that genetic factors affected fatty acid composition of carcass fat in Japanese Black Wagyu cattle (Oka et al., 2002). Similarly, genetic parameters were estimated in a study for fatty acid composition of subcutaneous beef fat of 1,573 animals which were the progeny of 157 sires across seven breeds (Kelly et al., 2013). Results concluded that much of the genetic variation in fatty acid composition was related to changes in fatness and as such selection for decreased fatness at a given weight

may cause a decrease in the proportions of MUFA in the subcutaneous fat and an increase in the proportions of saturated fatty acids (Kelly et al., 2013).

Smith et al. (2009) showed that miniscule differences exist in fatty acid composition between *Bos indicus* and *Bos taurus* cattle and that diet and time on feed are much more important determinants of beef fat content and fatty acid composition than breed type. In contrast to this study, Laborde et al.(2001) compared the fatty acid composition of two different *Bos taurus* breeds, Simmental and Red Angus. Results showed that the total lipid concentration of myristoleic acid (14:1), palmitoleic acid (16:1), and vaccenic acid (18:1n-7), and n-6 to n-3 fatty acid ratio, was greater ($P <$ 0.05) in Simmental than Red Angus (Laborde et al., 2001). These results coincide with a study examining variation of delta-9-desaturase values in dairy cattle which showed that delta-9-desaturase indices observed for Jersey and Brown-Swiss cows were lower compared with Holstein (Soyeurt et al., 2008). This result may help explain differences observed between breeds with regard to fatty acid composition.

Feeding Varying Dietary Supplements to Reduce the Saturation of Fatty Acids

The prevention of biohydrogenation and the formation of *trans* fats are important because complete biohydrogenation results in the production of stearic acid (C18:0). Vaccenic acid acts as an intermediate in the pathway of biohydrogenation of both linoleic and α-linolenic acid. Vaccenic acid represents about 60-70% of the *trans* fatty acid in milk and meat of ruminant animals (Emken, 1995). Mosley et al. (2002) showed that *trans* 18:1 isomers can also be formed in the rumen from oleic acid.

Rumen diets low in effective fiber can induce milk fat depression which results in an increase in milk fat content of *trans* fatty acids, specifically an increase in *trans*-10 18:1 (Griinari et al., 1998). Griinari et al. (1998) proposed that under certain dietary conditions the pathways of rumen biohydrogenation were altered to produce unique fatty acid intermediates that were potent inhibitors of milk fat synthesis. Baumgard et al. (2002) demonstrated that *trans*-10, *cis* 12 conjugated linoleic acid was a potent inhibitor of milk fat synthesis. With lipolysis being a prerequisite of biohydrogenation, it stresses the importance of inhibiting the ruminal microorganisms responsible for lipolysis so these fatty acid intermediates will not be produced.

Gulati et al. (1997) found that extruded oils provided about 15.0% protection while whole oil seeds provided about 40% protection to ruminal fats, as determined by *in vitro* assays. Harvatine and Allen (2006) examined the correlation between the rates of fatty acid biohydrogenation and passage through the rumen. They reported that passage rates of 16:0, 18:0 and total 18-carbon fatty acids were linearly decreased with increasing degree of unsaturation. Thus, increasing unsaturated fatty acids increased the extent of biohydrogenation and decreased the extent of 18:1 and *trans*-18:1. There have been a number of studies examining methods in reducing lipolysis and biohydrogenation by supplementing ruminal diets with different types of forages, fish oil, plant oils, and oil seeds (Ashes et al., 1992; Weill et al., 2002) with each of these studies having varying degrees of success.

Fish Oil

Eicosapentaenoic and docosahexaenoic acid are essential n-3 fatty acids and are the conversion products of α-linolenic acid. Animals are incapable of producing n-3 fatty acids. Cold-water fish are good sources of EPA and DHA (Kris-Etherton et al., 2000). These fatty acids are independently associated with an increase in LDL oxidation (Leigh-Firbank et al., 2002), which is why fish oil has been so extensively explored as a means to prevent lipolysis and biohydrogenation.

Leigh-Firbank et al. (2002) demonstrated the independent qualities of EPA and DHA by conducting a study where 55 men completed a double blind placebo controlled cross over study. The individuals that participated in the study either consumed 6 g of fish oil or 6 g of olive oil, which acted as the placebo, for two 6 wk periods. Docosahexaenoic acid was shown to be independently associated with the rise in LDL cholesterol. Eicosapentaenoic acid was shown to be separately associated with the reduction in fasting, postprandial triacylglycerol, and postprandial non-esterified fatty acid levels. These effects brought on by the consumption EPA and DHA aid in decreasing the risk of ischemic heart disease. In a physician's health study involving 25,551 males, consumption of one or more servings of fish per week was associated with a 52% lower risk of sudden cardiac death when compared to one fish meal/month (Albert et al., 2002).

Chow et al. (2004) investigated the effects of fish oil on biohydrogenation and lipolysis. They found that fish oil did not have a complete effect on ruminant biohydrogenation of linoleic and linolenic acid. The lipolysis of EPA and DHA was not

affected; however, biohydrogenation of EPA and DHA was reduced significantly with increasing fish oil. Linoleic and linolenic acid appeared to disappear to the same extent. Stearic acid, the end product of biohydrogenation, was significantly lower when fish oil was supplemented. These results indicate that only the final stages of the biohydrogenation pathway for linoleic and linolenic acid were inhibited by fish oil.

The pathway for the biohydrogenation, shown in Figure 1.2, of linoleic acid consists of three steps. In the first step *cis*-9, *trans*-11 conjugated linoleic acid is produced due to isomerization. Conjugated linoleic acid is then hydrogenated to produce *trans*-11 octadecanoic acid (vaccenic acid). Finally vaccenic acid is hydrogenated into stearic acid, which is the final product of the biohydrogenation process (Kepler et al., 1966; Harfoot et al., 1973; Kemp et al., 1984). Biohydrogenation of linolenic acid, also shown in Figure 1.2, produces *cis*-9, *tans*-11, *cis*-15 C18:3 and *trans*-11, *cis*-15 C18:2 as intermediates. The intermediates are progressively hydrogenated into vaccenic acid and finally to stearic acid as with the pathway for linoleic acid (Kemp et al., 1984).

Wasowska et al. (2006) further investigated the influence of fish oil and EPA and DHA alone or in combination with linoleic and linolenic acid on ruminal biohydrogenation. In their study both mixed ruminal microorganisms and pure cultures of *B. fibrisolvens* were used. The results were similar to that of Chow et al. (2004) in that they showed that fish oil inhibited the final step of biohydrogenation of both linoleic and linolenic acid, in turn causing an accumulation of a number of intermediates. Wasowska et al. (2006) showed that fish oil incubated with linoleic and linolenic acid caused accumulation of vaccenic acid from linoleic acid and *trans*-11, *cis*-15-18:2 from

linolenic acid. Non-esterified EPA and DHA were also added to mixed ruminal digesta at similar concentrations. Results showed that it did not replicate the effects of fish oil on linoleic acid metabolisms instead EPA and DHA inhibited linoleic acid isomerase activity of *B. fibrisolvens* (Wasowska et al., 2006).

Vegetable Oil

Along with fish oil, vegetable oil has also been extensively studied and used to decrease ruminal lipolysis and biohydrogenation. Rego et al. (2005) showed that cows supplemented with vegetable oil demonstrated a decrease in saturated fats and an increase in unsaturated fats in the milk fat content, suggesting that vegetable-derived oils may have a role in preventing ruminant biohydrogenation. When comparing sunflower oil to soybean oil, Rego et al. (2005) did not find a significant difference between fatty acid concentrations in milk fat. Their results showed that both oils decreased saturated fatty acids by 16.0% in comparison to the control cattle that were on pasture and being supplemented with 5 kg concentrate without either oil additive.

Sunflower Oil

Sackmann et al. (2003) investigated the effects of varying levels of forage and sunflower oil on ruminal biohydrogenation. The treatment groups for this study included grass hay fed at 12%, 24%, and 36% and sunflower oil at 2% and 4%. Dry matter intake was increased with increasing forage levels from 12% to 24%; until 36% forage was fed at which point there was a decrease in dry matter intake. In this study, sunflower oil did

not appear to alter dry matter intake. Linoleic biohydrogenation was found to be greater with 4% than with 2% concentration of sunflower oil, but dietary sunflower oil did not significantly alter ruminal biohydrogenation of oleic acid or linoleic acid at either level (Sackmann et al., 2003).

Linseed Oil

Weill et al. (2002) studied the protective effect that linseed oil had on fatty acids when introduced into a livestock diet. They introduced 5% of linseed oil into the diet of livestock. The results indicated that there was a decrease in the n-6/n-3 ratio by 54% in butter, 60% in meat, and 86% in eggs. Morris (2008a) demonstrated the effects of introducing linseed into beef cattle diets. The study reported that linseed increased α-linolenic acid and CLA content, and because of this increase the n-6/ n-3 ratio was decreased. The ratio was decreased by roughly half after each supplementation of linseed into the test cattle diets. The study did show, however, that there was little to no effect on saturated fat content in beef**.**

Varying Forages

Latham et al. (1972) found that lactating dairy cows fed a low fiber (20%) diet had lower levels of lipolysis and biohydrogenation of unsaturated fatty acids in the ruminal fluid than cows fed a diet high in fiber (44%). Sackmann et al. (2003) similarly determined that initial stage of ruminal biohydrogenation of oleic and linoleic acids were increased simultaneously as the levels of forage increased. When the biohydrogenation

of dietary linoleic acid to stearic acid is incomplete, it results in the yielding of several intermediates which can included *trans*- or *cis*-octadecenoic acids and conjugated linoleic acid isomers (Bauman et al., 2000). Increasing dietary forage levels showed a resulting increase on the duodenal flow of *trans*-11-vaccenic acid, an intermediate of linoleic acid, which in turn causes an increased outflow of intermediates.

However, the saturation of linoleic and α-linolenic acid has been reported to be substantially lower in ruminants fed red clover relative to grass silage (Al Mabruk et al., 2004; Van Dorland et al., 2008). This reduction of lipolytic activity has been shown to be associated with the enzyme polyphenol oxidase (Lee et al., 2004; Lourenço et al., 2005). Polyphenol oxidase works to deactivate lipase protein by covalently binding to nucleophilic sites forming a cross-linked protein polymer (Igarashi and Yasui, 1985). These findings suggest that feeding different types of forages can diversely alter ruminal biohydrogenation of fatty acids.

Technological Advances Used to Protect Lipids in the Rumen

The rapid accumulation of FFA produced by ruminal lipolysis limits the amount of fat that can be introduced into the daily diets of ruminants because of the inhibitory effects FFA have on the digestion of cellulose and fiber (Harfoot and Hazlewood, 1997). Lipids provide a high energy source and because of this, fat supplements are useful for meeting the energy requirements of animals, furthermore, it may be more cost effective for producers to provide energy as fat rather than as carbohydrates (Doreau and Chilliard, 1997). There have been several strategies developed in order to decrease or

bypass microbial biohydrogenation. Such strategies include encapsulation of unsaturated fatty-acids inside a microbe-resistant shell or alteration of fatty acid structure so as to help resist the action of ruminal microorganisms (Jenkins and Bridges, 2007).

Formaldehyde Cross-Linked Protein Casein

The treatment of dietary lipids with a layer of formaldehyde-treated casein protects lipids from ruminal lipolysis allowing for more unsaturated fatty acids to reach the small intestine for absorption. A number of studies have been conducted examining the effects of formaldehyde-protected fatty acids. Garrett et al. (1976) examined formaldehyde-protected linoleic acid in Holstein cattle and found that only 18-25% of the protected linoleic acid consumed was incorporated in the body tissue. However, studies by Faichney et al. (1973) and Hogan and Hogan (1976) showed that use of protected linoleic acid in sheep resulted in 50-60% of the protected linoleic acid consumed in the diet being stored in the body tissue.

Several research groups have looked at protected canola seed and its effects of the level of oleic acid in the milk on Holstein cattle (Ashes et al., 1992; Tymchuk et al., 1998; Delbecchi et al., 2001). Each study had varying levels of success with increasing levels of oleic acid in milk. Ashes et al. (1992) showed an increase from 23.8 to 29.2% in oleic acid in the milk. While the study conducted by Tymchuk et al. (1998) showed a 21.9% increase over the control, Delbecchi et al. (2001) showed similar results of a 22.0% increase of the oleic acid when fed protected canola seed over the control. Jenkins and Bridge (2007) compared several published studies that looked at the use of

protecting fatty acids through use of formaldehyde-cross-linked casein and found that with several types of fats that the casein gave protection from microbial degradation.

Calcium Salts

Calcium salts have also been used to help protect fat in the rumen. Fatty acids bind to calcium ions which in theory protects them from biohydrogenation. Research done by de Veth et al. (2005) compared the use formaldehyde-cross-linked casein and calcium salts. The study examined their use for protection of CLA from biohydrogenation and its applications in the dairy industry. Lipids bind to calcium ions making the lipids unavailable for bacterial uptake effectively preventing biohydrogenation.

The research done by de Veth et al. (2005) showed that calcium salts were effective at decreasing milk fat yield by 34.0% while only 3.2% of the calcium salt protected CLA was found in the milk. The study also showed slightly better results with the formaldehyde-cross-linked protected CLA and that it was effective in decreasing milk fat yield by 44.0% while 7.0% of the formaldehyde-protected CLA was found in the milk. Neither type of fat protection methods resulted in a decrease in milk yield. This study demonstrated that both calcium salts and formaldehyde are both effective to a degree in decreasing milk fat yields.

Harvatine and Allen (2006) also looked at the use of calcium salts as an inhibitor of biohydrogenation and found that the calcium salts did not have any effect on the protection of PUFA. Based on Harvatine and Allen (2006) and de Veth et al. (2005),

formaldehyde-protected CLA supplementation is currently the optimal method for use in the dairy industry at lowering milk fat yield.

Amide Protected Lipids and Lipid Encapsulation

Perfield II et al. (2004) studied two different methods used to protect CLA from microbial degradation in the rumen. These two methods include amide-protected CLA and lipid encapsulated CLA. The amide-protected CLA and the encapsulated CLA were both incorporated into the diets of Holstein cattle and compared to a control lacking conjugated linoleic acid supplementation. Both types of CLA protected supplements showed similar decreases in milk fat with a 21% decrease in the cattle fed the amide-protected CLA and 22% in the lipid encapsulated CLA. The amount of total *trans*-10, *cis*-11 CLA that was transferred into the milk fat was the same for both amide protected (7.1%) and the lipid encapsulated (7.9%). Both types of protection methods of CLA resulted in decreases in secretion of all milk fat fatty acid constituents. However, fatty acids containing ≤ 16 carbons were reduced slightly more than other fatty acids. Each method did show gradual decrease in milk secretion over the course of the study while having no decrease in dry matter intake or milk fat, but the decrease was not significant enough to consider for application in the industry.

Supplementing Glycerol in the Rumen

Crude glycerin is a major byproduct in the production of biodiesel. Glycerin's availability and nutritional properties has made it an attractive choice for use in ruminant

animals as a feed supplement (DiLorenzo and Galyean, 2010). Corn distillers dried solids, which are used as animal feed, have been shown to contain appreciable concentrations of glycerol (Wu, 1994). Feedlot cattle have shown to consume more feed and gained more weight when fed diets supplemented with 2 to 8% crude glycerin than when fed diets containing no added glycerin (Parsons et al., 2009). However, when cattle were fed diets supplemented with ≥12% crude glycerin, rumen digestibility was inhibited (Parsons et al., 2009).

In lactating dairy cows, an effect was not apparent on milk production or feed intake when lactating dairy cows were fed diets supplemented with 5 or 10% refined glycerol, the main component of glycerin, although when cattle were fed a diet supplemented with 15% glycerol a slight decrease in intake was observed (Donkin et al., 2009). Conversely, Gunn et al. (2010) demonstrated improved intake and weight gain in finishing lambs fed diets containing up to 15% added glycerin. Additionally, Gilbery et al. (2010) examined the effects of glycerol supplementation on dry matter intake in steers fed finishing diets. Holstein steers were fed one of four treatments of glycerol (0%, 6%, 12%, and 18%). Results showed that glycerol exceeding 6% of dietary dry matter reduced dry matter and natural detergent fiber intake. Ruminal digestion of crude protein and natural detergent fiber were decreased as dietary glycerol increased in the finishing diet.

Research by Krueger et al. (2010) evaluated two levels of glycerol (2% and 20%) *in vitro* and its inhibitory effects on ruminal lipolysis in mixed culture. Their study demonstrated that feeding glycerol at these two levels resulted in a 48% and 77%

reduction in FFA accumulation. Results from this also indicated that supplementing glycerol at 20% of the total ration may negatively affect digestion of the fibrous fraction of the feed. However, when fed levels of glycerol less than 20% there did not appear to be any affect on the digestion of natural detergent fiber. Another study done on supplemental glycerol has shown that glycerol supplemented up to 10% in the daily diet does not have any effect on feed intake or performance of finishing beef cattle or lactating dairy cows (Strompl et al., 1999; Kerley, 2007). Edwards et al. (2012), reported a ($P < 0.05$) more than 80% reduction of mixed culture lipolytic rates *in vitro* when supplemented with glycerol with no difference in level of inhibition observed whether supplemented with 6 or 20% (v/v) glycerol ($P < 0.05$), further confirming the earlier work of Krueger et al. (2010). Results from these studies suggest that supplementing glycerol at around 8 to 15% of diet dry matter would achieve effective reductions in lipolysis without adversely affecting rumen dry matter digestion. However, linear increases in ruminal propionate and butyrate and linear reductions in acetate to propionate ratio with increasing glycerol doses have been observed (Wang et al., 2009; Krueger et al., 2010).

These changes in pattern of VFAs with increasing glycerol supplementation suggests that long-term feeding of glycerol may result in the selection and enrichment of glycerol-fermenting microbes such as *Megasphaera elsdenii* and *Selenomonas rumiantium*. The enrichment of these bacteria would result in the rapid fermentation of glycerol resulting in a decrease or the complete elimination of the inhibition effect on lipolytic activity. The enrichment of these bacteria may only allow for glycerol

supplementation to serve as only a short term strategy to inhibit lipolytic activity and the saturation of fatty acids. Therefore, this strategy may be effective to significantly change the fatty acid composition in ruminal animals due to its short term use.

Monensin Supplementation

Monensin is a carboxylic polyether ionophore which is produced by the fermentation of *Streptomyces cinnamonensis* (Russell, 2002). Monensin is commonly used in the dairy industry to increase milk production and improve energy balance; however, it has been shown to inhibit the growth of Gram-positive bacteria (Bauman et al., 2000). Bacteria that contribute to the saturation of fatty acids considered in the current study are Gram-positive bacteria with the exception of *A. lipolyticus.* Because of monensin's ability to inhibit some of the major bacterial contributors to ruminal lipolysis and biohydrogenation, it has additionally been examined for use as a means to enrich meat and milk with unsaturated fatty acids.

Fatahnia et al. (2010) reported that supplementing diets with monensin in lactating dairy cattle significantly increased concentrations of unsaturated fatty acids in milk fat. Fatahnia (2010) also showed that monensin reduced the percentage of short-chain fatty acids but had no effect on medium- and long-chain fatty acids with overall milk fat percentage showing no effect with monensin supplementation. Similarly, Da Silva et al. (2007) showed that monensin supplementation increased concentrations of *cis*-9 and *trans*-11 conjugated linoleic acid and also decrease the concentrations of saturated fatty acids in milk fat. Several studies have conversely shown that

supplementation with monensin depressed milk fat percentage (Phipps et al., 2000; Da Silva et al., 2007; Alzahal et al., 2008). This effect has been suggested to be due to an incomplete biohydrogenation as a result of monensin's inhibition of hydrogenating bacteria causing an increase in *trans*-10, *cis*-12 conjugated linoleic acid, which has found to be a potent inhibitor of *de novo* synthesis of fatty acids (Bauman and Griinari, 2001). The study by Fatahnia (2010) suggests that the reduced percentage of short-chain fatty acids may also be contributed to an inhibition in *de novo* fatty acid synthesis due to an increase in the biohydrogenation intermediate *trans*-10, *cis*-12. These studies demonstrate monensin can effectively be used as a means to significantly enrich milk with unsaturated fatty acids; however, subsequently milk fat percentage could negatively be affected.

Immunogenic Inhibition of Ruminal Bacteria

The present study focuses on the development of a method to immunogenically inhibit ruminal bacteria responsible for the saturation of fatty acids. Williams et al. (2009) developed a vaccine against ruminal methanogens in an attempt to alter the composition of archaeal populations and reduce methane production from ruminal animals. Williams et al. (2009) was successful at increasing specific IgG titers in plasma, saliva, and rumen fluid using a designed vaccine targeting species/strains of methanogens in sheep and altering the composition of the methanogen population. However, the vaccine did not appear to reduce methane output compared to the control sheep which did not receive the vaccine.

Unlike the production of methane, lipolysis is an extracellular process and thus the lipase enzyme would have more potential to come into contact with an inhibitory antibody. Williams et al. (2009) was successful at producing antibodies against specific bacteria, however, since the process of methane production is intracellular it would be difficult for the antibody to interfere with this process and may be why a reduction in methane production was not observed. Using the same concept to produce antibodies against key lipase producing bacteria, we hypothesize that we will be successful at reducing lipolytic activity and the saturation of fatty acids.

CHAPTER II

CHARACTERIZATION OF RUMINAL LIPASE-PRODUCING BACTERIA AND THEIR LIPOLYTIC ACTIVITIES *IN VITRO*

Introduction

Dietary fatty acids are metabolized as fuel for oxidative phosphorylation; during a time of energy surplus they are stored as triacylglycerols (TAG) or rapidly incorporated into plasma phospholipids, high-density lipoprotein particles or cell membranes (Rennison and Van Wagoner, 2009). Lipid metabolism is initiated by hydrolysis of ester linkages in triglycerides, phospholipids, and glycolipids. Hydrolysis and biohydrogenation ruminally are driven by microbial metabolic activity (Shorland et al., 1955; Viviani, 1970). Ruminal microorganisms in the rumen reduce and hydrogenate the double bonds of unsaturated fatty acids, transforming unsaturated fat into saturated fat (Doreau and Chilliard, 1997). Lactating dairy cows, on average, consume 300 g of linoleic acid daily, but of this about 40 g reaches the small intestine unsaturated (Jenkins and Bridges, 2007). Thus, saturated fats accumulate in the meat and milk of ruminants.

The extent to which an unsaturated fatty acid is subject to ruminal biohydrogenation is determined by the fat source, retention time, and characteristics of the microbial population (Allen, 2000). Several different types of microorganisms found in the rumen that are capable of hydrolyzing dietary lipids. This study focuses on two bacteria that recently have been found to demonstrate substantial lipolytic activity *in vitro*: *Propionibacterium avidum* and *P. acnes* (Edwards et al., 2013). *Butyrivibrio*

fibrisolvens 49 and *Anaerovibrio lipolyticus* 5s also were investigated due to previous research that documented them as major contributors to ruminal lipolysis (Polan et al., 1964; Henderson, 1971; Henderson and Hodgkiss, 1973; Prins et al., 1975)

Crude glycerin is a major by-product in the production of biodiesel, and because of its availability and nutritional properties, it has become an attractive choice for use as a feed supplement for beef cattle (DiLorenzo and Galyean, 2010). Feedlot cattle consumed more feed and gained more weight when fed diets supplemented with 2 to 8% crude glycerin than when fed diets containing no added glycerin (Parsons et al., 2009). Additionally, Krueger et al. (2010) supplemented 2 or 20% glycerol *in vitro* to mixed populations of ruminal microbes and found that rates of FFA accumulation were reduced by 48 and 77%, respectively. Lipase-producing bacteria efficiently hydrolyze lipids, thereby liberating the free carboxyl group required for the initial step of biohydrogenation (Kepler et al., 1970). Thus, these studies show that supplementing glycerol in ruminal diets can increase carcass quality by increasing overall carcass weight and reducing the deposit of saturation of fatty acids in their meat.

Corn distillers' dried soluble, a common animal feed, have been shown to contain glycerol at 3-10% (Wu, 1994). However, grain-based diets provide substantial quantities of free glucose to ruminal bacteria (Huntington et al., 2006), which may affect rate of lipolysis. Cereal grains contain 57 to 77% of dry matter as starch, while other feedstuffs such as grasses and legumes contain 2 to 20% starch (Huntington et al., 2006). Past studies have shown that a high dietary concentrations of fermentable starch are associated with increased production of organic acid, increased production of microbial

protein, decreased fiber digestion, decreased ammonia concentrations, and decreased acetate:propionate (Poore et al., 1993; Martin et al., 1999; Philippeau et al., 1999).

We hypothesized that supplemental glucose and glycerol *in vitro* would affect the lipolytic response of pure and mixed cultures of ruminal bacteria. Therefore, we predict results from this study will provide functional characterization to pure cultures of ruminal lipase-producing bacteria and also define their contribution to the overall lipolytic activity in the rumen by comparing their activity to mixed cultures in the presence of these energy substrates.

Materials and Methods

Treatments

This experiment tested the hypothesis that media supplemented with glucose and glycerol could be used to characterize mixed ruminal cultures and pure cultures of *Anaerovibrio lipolyticus* 5s, *Butyrivibrio fibrisolvens* 49, *Propionibacterium avidum*, and *P. acnes*. Energy substrates glucose (0 and 0.5%) and glycerol (0 and 2%) were added to media in a 2 × 2 factorial design. Substrate concentrations were incorporated based on the past research of Krueger et al. (2010) and Cotta and Hespell (1986).

Microbial Preparation and Handling Procedures of Cultures Used in this Study

The mixed bacterial population used in this study was obtained from fresh rumen fluid collected from a cannulated cow grazing on predominantly rye grass pasture. Rumen fluid was strained though a nylon paint strainer into a pre-warmed insulated

container that had been flushed with warm water prior to sample collection. The container was filled completely (approximately 500 mL), capped and transported to the lab. Upon arrival at the laboratory, CO_2 was bubbled through the rumen fluid to keep it in an anaerobic state until its use as inoculum (within 30 min of collection). The cow was cared for according to procedures approved by Southern Plains Agricultural Research Center's Animal Care and Use Committee (Protocol #2011004).

Pure cultures of *Anaerovibrio lipolyticus* 5s and *Butyrivibrio fibrisolvens* 49 were obtained from Dr. Jay Yankee, Agriculture-Agri Food Canada. Strains of *Propionibacterium avidum* and *Propionibacterium acnes* were previously isolated from the rumen of a pastured cow (Krueger et al., 2008). For long-term preservation of pure cultures, bacteria were stored in 20% anaerobic glycerol at -80ºC. Upon removal from storage, each bacterial strain was revived via two consecutive 24 to 48 h cultures in 10 mL standard anaerobic broth supplemented with 2% pre-sterilized olive oil. Tubes were incubated at 39°C and agitated horizontally at 35 rpm in an Innova™ Model 4000 Shaking Incubator (New Brunswick Scientific Co., Inc., Edison, NJ).

Standard Anaerobic Medium Formulation

All chemicals were purchased from Sigma-Aldrich 41 (Milwaukee, WI) unless otherwise noted. Standard anaerobic broth medium contained (per liter): 292 mg each of K_2HPO_4 and $KHPO_4$, (JT Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ), 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_20$, 4,000 mg Na_2CO_3, 600 mg cysteine-HCl (J.T. Baker), 10 g trypticase (BBL Microbiology

Systems, Cockeysville, MD), 2.5 g yeast extract (Difco; Becton, Dickinson and Company, Sparks, MD) branched-chain fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), 2 μg hemin, vitamin mix (20 mg each thiamine, pantothenate, nicotinamide, pryridoxine HCl, riboflavin, 1 mg *p*-aminobenzoic acid, 0.5 mg biotin, 0.5 mg folic acid, 0.2 mg vitamin B-12, and 0.5 mg lipoic acid), and trace minerals (Cotta and Russell, 1982). Media were boiled to displace dissolved oxygen and then cooled on ice while being saturated with continuous flow O_2-free CO_2 for 20 min. Media were distributed to 18 x 150 mm glass walled tubes using the anaerobic Hungate technique and capped (Bryant, 1972). Media was immediately autoclaved and cooled before use.

Characterization of Ruminal Lipase Producing Bacteria

Tubes were inoculated with 0.2 mL of pure populations, from revived cultures, and mixed populations, from a cannulated cow, to determine their lipolytic activity when cultured in the presence and absence of 2.0% glycerol and 0.05% glucose. Tubes used to culture the bacteria were pre-loaded with 0.3 mL olive oil, 21 g glass beads, and 6.0 mL anaerobic medium. Tubes were incubated at 39°C in an Innova™ Model 4000 Shaking Incubator (New Brunswick Scientific Co., Inc., Edison, NJ) at 90 rpm for 48-h. Concentrated HCl (0.5 mL) was added to stop growth and lipolytic activity following inoculation (zero time controls) and after incubations. Fatty acids were extracted and quantified by the colorimetric procedures of Kwon and Rhee (1986).

Statistic Analysis

Test for the effects of the different treatments was performed using a two-way general ANOVA (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a least squares differences (LSD) separation of means ($P < 0.05$). The model included main effects of varying glucose, glycerol, and their interaction; $Y_{ijk} = \mu + glucose_i + glycerol_j + (glucose \times glycerol)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, glucose and glycerol are the fixed effects of the experimental treatments (i = 1 to 2; j = 1 to 3), glucose × glycerol is their interaction, and ε_{ijk} is the error term.

Results

Media Free Fatty Acids

The addition of glucose decreased ($P < 0.05$) FFA release by *A. lipolyticus* 5s, *B. fibrisolvens* 49, and *P. acnes* (Table 2.1). However, glucose increased ($P < 0.05$) FFA release by *P. avidum* and the mixed population. Conversely, glycerol increased ($P < 0.05$) FFA release for all four pure culture bacterial species, whereas glycerol reduced ($P < 0.05$) FFA release in the mixed population. Mixed population and *P. acnes* displayed a ($P < 0.05$) glucose × glycerol interaction. Glycerol increased ($P < 0.05$) FFA release by *P. acnes* even in the presence of glucose but to a greater extent when glucose was absent in media. Conversely, glycerol decreased ($P < 0.05$) FFA release by mixed culture but to less of an extent when glucose was present.

Table 2.1 Least square means for media free fatty acids of bacteria grown in the presence of olive oil supplemented with glycerol and/or glucose.

	Specific activity (nmol free fatty acid/mg protein per h)[a]									
	Mixed culture		*A. lipolyticus* 5s		*B. fibrisolvens* 49		*P. avidum*		*P. acnes*	
	0% Glucose	0.05% Glucose	0% Glucose	0.05% Glucose	0% Glucose	0.05% Glucose	0% Glucose	0.05% Glucose	0% Glucose	0.05% Glucose
0% Glycerol	138.5[b]	380.7[a]	77.4	28.6	93.3	59.6	0.01	685.3	74.1b[c]	54.7[c]
2% Glycerol	13.1[c]	37.8[c]	182.7	120.8	312.0	209.8	38.4	816.1	264.0[a]	129.4[b]
P-values										
Glucose	0.001		0.022		0.029		<0.001		0.003	
Glycerol	<0.001		0.001		0.001		0.004		0.001	
Interaction	0.006		0.745		0.211		0.064		0.014	
SEM	29.4		19.7		28.7		21.5		18.5	

[a,b,c] Least square means within culture group with common superscripts do not differ ($P > 0.05$).

Discussion

Culture studies have found *A. lipolyticus* to be present in the rumen at around 10^7/mL (Prins et al., 1975), suggesting a dominant functional role with regards to ruminal lipolytic activity. The lipase produced by *A. lipolyticus* appears to be more functionally adapted to hydrolyzing diacylglycerols than TAG (Henderson, 1970; Henderson, 1971; Henderson and Hodgkiss, 1973). Compared to the other bacteria included in this study, *A. lipolyticus* has
limited capacity for fermenting carbohydrates; only glycerol, fructose and ribose are fermented with acetic, propionic, and succinic acids being formed (Henderson, 1975).

Butyrivibrio species also have been known to play a major role in the ruminal hydrolysis of dietary lipids, contributing mainly but not exclusively to the hydrolysis of galactolipids and phospholipids (Lourenço et al., 2010). Some strains of *B. fibrisolvens* are auxotrophic for fatty acids, so hydrolysis of ruminal lipids may provide fatty acids needed for cell membrane synthesis (Hazlewood et al., 1980). Unlike *A. lipolyticus*, *B. fibrisolvens* can obtain energy for growth by fermenting a wide variety of sugar and amino acid substrates (Cotta and Forster, 2006).

Very little is known about either specie of *Propionibacterium* or their role in rumen metabolism. Both *Propionibacterium* species are similar to *B. fibrisolvens* in that they have the ability to utilize a variety of amino acids as energy sources (Ferguson and Cummins, 1978; Holland et al., 1979). However, Greenman et al. (1981), demonstrated that *P. avidum* was more nutritionally adaptable at utilizing glucose as a substrate than *P. acnes*.

Lipolytic activities from pure cultures were vastly different from that of mixed culture in the presence of glucose and glycerol (Table 2.1). All pure culture bacteria displayed exceedingly higher rates of lipolytic activity when glycerol was present as an energy substrate, while mixed cultures appeared to be glycerol sensitive. These results coincide with other studies showing that glycerol can be used as an effective means to inhibit lipolytic activity in the ruminal mixed cultures (Krueger et al., 2010; Edwards et al., 2012). The opposite effect when glycerol was used as the energy substrate, increasing ($P < 0.05$) activity of pure cultures and decreasing ($P < 0.05$) activity of mixed cultures. Glucose increased ($P < 0.05$) the lipolytic activity of *P. avidum* and mixed cultures while it appeared to inhibit the lipolytic activity of *A. lipolyticus* 5s, *B. fibrisolvens* 49, *and P. acnes*. As indicated previously, *P. avidum* is nutritionally diverse (Greenman et al., 1981), which may explain why FFA release was increased in the presence of both glucose and glycerol. However, these results clearly show that mixed culture and pure cultures of well know ruminal lipase-producing bacteria were different when grown in the presence of these two energy substrates.

Results further indicate the inability of *A. lipolyticus* 5s to utilize glucose as substrate for growth. However, these results suggest that while glucose is not utilized for the growth of *A. lipolyticus* 5s, it may be interfering with its production of a functional lipase. This was demonstrated when the utilizable substrate, glycerol, was administered in combination with glucose, which showed lipolytic activity was still slightly reduced for *A. lipolyticus* 5s.

In conclusion, this study indicated that the lipase activity of *P. avidum* in the presence of glucose contributed more to lipolysis of dietary lipids than *A. lipolyticus* 5s, *B. fibrisolvens* 49, or *P. acnes*. However, unlike mixed cultures, *P. avidum* was unaffected by glycerol, thus there may be an important glycerol-susceptible bacterial species contributing to ruminal lipolysis that has yet to be identified.

CHAPTER III

PRESUMPTIVE RUMINAL *CLOSTRIDIUM* SPECIES IMPLICATED AS CONTRIBUTORS TO OVERALL RUMINAL LIPOLYTIC ACITIVITY

Introduction

Nutritional approaches are currently being sought to naturally fortify milk and meat with health-promoting MUFA and PUFA (Antonigiovanni et al., 2003; Decker and Park, 2010). Biohydrogenation is a ruminal process that almost quantitatively saturates FFA, thus limiting the availability of unsaturated fatty acids for absorption and assimilation. In order for biohydrogenation to occur, FFA must first be hydrolyzed from their TAG precursors (Harfoot and Hazlewood, 1997), a process known as lipolysis. To preserve dietary unsaturated fatty acids in the rumen, key contributors to ruminal lipolysis need to be further identified and characterized.

A. lipolyticus 5s, *B. fibrisolvens* 49, *P. avidum,* and *P. acnes* are recognized as prominent lipase-producing bacteria in the rumen. Additionally, certain lipolytic clostridial species have been isolated from dilute rumen populations (van Gylswyk and van der Toorn, 1987; Jarvis et al., 1998; Krueger et al., 2008); and been shown to be present in the rumen at high numbers (up to 10^5 to 10^{10} cells/mL); however, little is known about their overall contribution to ruminal lipolysis. Several *Clostridium* species from rumen and non-rumen environments produce lipase and these include *C. lundense* (Cirne et al., 2006), *C. tetanomorphum, C. tetani,* (Wilde et al., 1989), *C. botulinum, C. aurantibutyricum, C. novyi, C. sporogenes* (Cato et al., 1986) and *C. ghonii* (Sneath,

1986). Because of the diversity of this group it is reasonable to hypothesize that lipase-producing clostridia may collectively constitute an appreciable amount of lipolytic activity in the rumen. Clostridia are rod shaped, obligate anaerobic bacteria generally capable of producing heat-resistant endospores; this latter characteristic allows assessment of the overall contribution of heat-resistant clostridia to lipolysis in mixed populations of rumen bacteria.

Glycerol supplemention, *in vitro,* at 6% decreased lipolytic activity of mixed populations of ruminal microbes by more than 80%, relative to non-supplemented controls (Edwards et al., 2012). However, the previous chapter demonstrated that lipolysis by pure cultures of *A. lipolyticus* 5s, *B. fibrisolvens* 49, *P. acnes* or *P. avidum* were not inhibited by glycerol. The mechanism responsible for the inhibition of lipolysis by mixed populations has not been defined, but the absence of glycerol-sensitive lipolytic activity in pure cultures of *A. lipolyticus* 5s, *B. fibrisolvens* 49, *P. acnes,* and *P. avidum* suggests that other important glycerol-susceptible ruminal lipolytic bacteria have yet to be identified and characterized. Accordingly, the objectives of the present study were to characterize the lipolytic contribution and glycerol susceptibility by heat-treated and non-heat-treated ruminal microbes.

Materials and Methods

Treatments

Two trials were performed to examine the role that heat-resistant presumptive *Clostridium* play in ruminal lipolysis. Trial 1 consisted of heat-treated rumen

populations, which then were adapted to a basal rumen fluid/olive oil medium in four consecutive, 48 h incubations. A control population also was adapted to the media but did not receive the heat treatment. Following heat treatment and adaptation, each population was used as inoculum of tubes without or with 6% glycerol for characterization and incubated for another 48 h. Samples of the heat-treated and non-heat-treated population were taken before and after adaptation to the media as well as after glycerol supplementation for denaturing gradient gel electrophoresis (DGGE) analysis. Additionally, samples of pure cultures of *C. sporogenes, A. lipolyticus, B. fibrisolvens*, and *P. acnes* were taken for DGGE analysis for comparison to the mixed populations.

Trial 2 consisted of two ruminal populations that were adapted to the olive oil medium as before; however, following the final incubation, one population received the heat treatment and one served as the control and did not receive the treatment. Again, both treatments were used as inoculum and incubated for a final 48 h with or without 6% glycerol.

Microbial Preparation and Handling Procedures

The mixed bacterial populations used in this study were obtained from fresh rumen fluid collected from a cannulated cow grazing on predominantly rye grass pasture. Rumen fluid was strained though a nylon paint strainer (Leyendecker et al., 2004) into a pre-warmed insulated container that had been flushed with warm water (approximately 39^{o}C) prior to sample collection. The container was filled completely

(approximately 500 mL), capped and transported to the lab. Upon arrival at the laboratory, CO_2 was bubbled through the rumen fluid to maintain anaerobiosis until its use as inoculum (within 30 min of collection). The cow was cared for according to procedures approved by Southern Plains Agricultural Research Center's Animal Care and Use Committee (Protocol #2011004).

Rumen Fluid Based Basal Medium Formulation

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The basal rumen fluid/olive oil medium contained (per liter) 100 mL clarified rumen fluid, 150 mL mineral mix 1, 150 mL mineral mix 2, 1 mL of 0.1% resazurin, 4 g $NaHCO_3$ and 0.5 g cysteine-HCl per liter. Mineral mix 1 contained (per liter) 6 g K_2HPO_4 and mineral mix 2 contained (per liter) 6 g KH_2PO_4, 12 g $(NH_4)_2SO_4$, 12 g NaCl, 1.2 g $MgSO_4*7H_2O$, 1.2 g $CaCl_2 \cdot 6H_2O$ and 6 g $CaCl_2$. The medium was prepared by boiling to remove dissolved O_2 and cooled on ice under a continuous flow of 100% CO_2. The cooled medium was distributed (6 mL/tube) using the anaerobic Hungate technique (Bryant, 1972) into 18 x 150 mm glass tubes pre-loaded with 5.0% olive oil and 21 g glass beads. The tubes were closed immediately with rubber stoppers and crimp tops. Media were immediately sterilized, cooled to room temperature, and supplemented with previously sterilized and degassed olive oil to achieve 6% olive oil (vol/vol) at the start of each incubation.

Determination of Lipolytic Contribution of Mixed Populations of Rumen Bacteria

In Trial 1, mixed populations of ruminal microbes were first subjected to a physical enrichment for mesophilic sporeformers via a 10 min 80^{o}C heat treatment (Krieg, 1981). The heat-treated ruminal fluid was inoculated (0.2 mL) into basal rumen fluid/olive oil medium and cultured in triplicate over the course of four, consecutive 48 h batch cultures under 100% CO_2 gas at 39^{o}C in an Innova™ Model 4000 Shaking Incubator (New Brunswick Scientific Co., Inc., Edison, NJ) at 30 rpm. Each culture series was conducted in triplicate. Tubes of each successive series were inoculated with 0.2 mL from cultures of the previous series. Triplicate sets of non-heat-treated ruminal populations were cultured likewise which served as controls. Free fatty acids were measured colorimetrically (Kwon and Rhee, 1986) at the end of each incubation series and rates of FFA accumulation were calculated as total FFA produced per 6 mL per-h (Edwards et al., 2012). At the end of the fourth consecutive culture series, 0.2 mL from each of the heat-treated or non-heat-treated cultures were transferred into tubes containing basal rumen fluid/olive oil medium supplemented without or with 6% glycerol and incubated as prior in a final fifth culture series.

In Trial 2, mixed populations of ruminal microbes were first adapted to growth in the basal rumen fluid/olive oil medium via four consecutive batch cultures. Each culture series was incubated as describe in Trial 1. Accordingly, tubes containing basal rumen fluid/olive oil medium were each inoculated, in triplicate, with 0.2 mL fresh ruminal fluid. Each culture was conducted in triplicate. Tubes of each successive series were inoculated with 0.2 mL from cultures of the previous series. After the fourth incubation

series, two of the triplicate tubes were selected at random, with one tube being subjected to 10 min heat treatment at 80^{o}C to provide inoculum enriched with mesophilic spore formers and the other tube used to provide non-heat-treated inoculum to provide controls. Aliquots (0.2 mL) from the heat-treated and non-heat-treated cultures were then inoculated into tubes containing fresh basal rumen fluid/olive oil medium and supplemented with or without 6.0% (v/v) glycerol and incubated in a fifth consecutive culture series as described previously. Free fatty acids were measured colorimetrically for each enrichment series (Kwon and Rhee, 1986).

DNA Extraction and DGGE

To assess the potential population changes following consecutive batch culture of heat-treated and non-heat-treated cultures and their response to glycerol administration, the populations from trial 1 were subjected to DGGE analysis. The DGGE also was performed on heat-treated and non-heat-treated rumen fluid used as the inocula in trial one to compare to populations after consecutive batch culture. Cell lysis and DNA extraction from sampled populations was achieved using a PowerSoil UltraClean MoBio Kit (MO BIO, Carslbad, CA, USA). Cells from a pure culture of *C. sporogenes* were processed simultaneously using the same method to ensure its effectiveness in cell lysis and DNA extraction from this Gram-positive, spore-forming bacterium. Genomic DNA was isolated from 1.0 mL of each sample with a QIAamp DNA Mini Kit (Qiagen Ltd., Crawley, UK) according to the method described in the kit. Cells from pure cultures of *A. lipolyticus* 5s, *B. fibrisolvens* 49, and *P. acnes* also were processed to ensure their

disappearance from the heat treated cultures. Samples were centrifuged for 10 min at 5000 x *g* and pellets were suspended in 180.0 µL of enzyme solution (20.0 mg/mL lysozyme, 20.0 mM Tris-HCl, pH 8.0, 2.0 mM EDTA) and 1.2% Triton-X 100 and incubated for 30 min at 37°C.

Denaturing gradient gel electrophoresis was performed according to previously reported method (Muyzer et al., 1993) with modification (Hume et al., 2003), using PCR primers targeted to conserved regions flanking the variable V3 region of 16S rDNA. Primers (50.0 pmol) were each mixed with Jump Start Red-Taq Ready Mix, according to methods described in the kit, and 250.0 ng of pooled (50.0 ng per chick) template DNA from each sample group. Amplifications were done on a PTC-200 Peltier Thermal Cycler (MJ Research; MJ Research, Inc., Waltham, MA, USA) with the following program: 1) denaturation at 94.9°C for 2 min; 2) subsequent denaturation at 94.0°C for 1 min; 3) annealing at 67.0°C for 45 sec; -0.5°C per cycle (touchdown to minimize spurious by-products (Don et al., 1991; Muyzer et al., 1993); 4) extension at 72.0°C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94°C for 1 min; 7) annealing at 58.0°C for 45 sec; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72.0°C for 7 min; 10) 4.0°C final.

Polyacrylamide gels (8.0% [v/v] acrylamide-bisacrylamide ratio 37.5:1) were cast with a 35 to 60% urea-deionized formamide gradient; 100% denaturing acrylamide with 7.0 M urea and 40% deionized formamide. Amplified samples were mixed with an equal volume of 2x loading buffer (0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol, and 70.0% [v/v] glycerol) and 4.0 µL placed in each sample well (16-well

comb). Gels were placed in a DCode Universal Mutation Detection System for electrophoresis in 1x TAE (20.0 mM Tris [pH 7.4], 10.0 mM sodium acetate, 0.5 M EDTA) at 59°C for 17 h at 60.0 V. Gels were stained with SYBR Green I (1:10,000 dilution) and fragment pattern relatedness determined with Molecular Analysis Fingerprinting Software, v.1.6, based on the Dice similarity coefficient and the un-weighted pair group method using arithmetic averages for clustering.

Statistical Analysis

Data were analyzed using a two-way general ANOVA (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a LSD separation of means ($P < 0.05$). The model included main effects of non-heat and heat treatments, 0 and 6% glycerol, and their interaction; $Y_{ijk} = \mu + \text{heat treatment}_i + \text{glycerol}_j + (\text{heat treatment} \times \text{glycerol})_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, heat treatment and glycerol are the fixed effects of the experimental treatments (i = 1 to 2; j = 1 to 2), heat treatment × glycerol is their interaction, and ε_{ijk} is the error term.

Results

Enrichment of Ruminal Lipase-Producing Bacteria

Rates of FFA accumulation during consecutive batch culture of non-heat-treated ruminal microbes did not differ ($P = 0.42$) between the first and second trials and did not differ ($P = 0.37$) across incubation series; therefore, data from non-heat-treated cultures in both trials were pooled and average rates of FFA accumulation are presented in Figure

3.1. Rates of FFA accumulation in consecutive batch cultures inoculated with heat-treated ruminal populations (Trial 1) were reduced ($P < 0.0001$) compared to those inoculated with non-heat-treated ruminal microbes (Figure 3.1).

Rates of FFA accumulation by heat-treated cultures from Trial 1 were reduced ($P < 0.0250$) by 82 % compared to non-heat-treated cultures (Table 3.1). In Trial 2, rates of FFA accumulation were reduced by 63% from non-heat-treated populations for populations that were culturally adapted prior to heat treatment. For both Trial 1 and Trial 2, there was a significant glycerol effect ($P = 0.036$ and 0.041). Presence of glycerol reduced the accumulation of FFA in both trials independent of the populations being adapted to the media or whether they were heat treated. There was no heat treatment x glycerol interaction for either of the trails ($P > 0.05$).

Denaturing Gradient Gel Electrophoresis

Heat-treated populations following adaptation (lanes 4 and 5) had fewer DGGE bands then non-heat-treated populations (lanes 1 and 2; Figure 3.2). Non-heat and heat-treated populations exhibited 55% similarity. Intensity and number of the bands varied between the glycerol and non glycerol-supplemented populations. Non-heat-treated populations supplemented without or with glycerol (lanes 4 and 5) were 78% similar, whereas heat-treated populations supplemented with or without glycerol (lanes 1 and 2) were 89% similar. There were more bands for the microbial population from rumen fluid (lanes 6 and 7) sampled prior to the enrichment phase of the experiment. The positive

Table 3.1 Effects of glycerol-supplementation on rates of free fatty acid accumulation in mixed populations of non-heat-treated and heat-treated ruminal microbes culturally adapted to growth in a basal rumen fluid/olive oil.

	Accumulation of free fatty acids (nmol $mL^{-1}h^{-1}$)							
	Non-heat-treated populations		Heat-treated populations					
Trial[a]	No glycerol	Glycerol	No Glycerol	Glycerol	SEM	Treatment	Glycerol	Interaction
One[b]	56.38	12.69	10.18	<0.01	10.70	0.025	0.036	0.156
Two[c]	41.19	0.33	15.11	10.24	9.39	0.415	0.041	0.092

All treatments were replicated in triplicate

[a]All populations were adapted to the basal rumen fluid/olive oil medium via four, consecutive 48 h batch cultures performed at 39°C. Tests for glycerol sensitivity were assessed during their fifth and final consecutive batch culture series in the basal rumen fluid/olive oil medium supplemented with or without 6% (vol/vol) glycerol.

[b]Heat-treated populations in Trial 1 were heat-treated prior to initiation of their cultural adaptation period.

[c]Heat-treated populations in Trial 2 were heat-treated at the end of their cultural adaptation period.

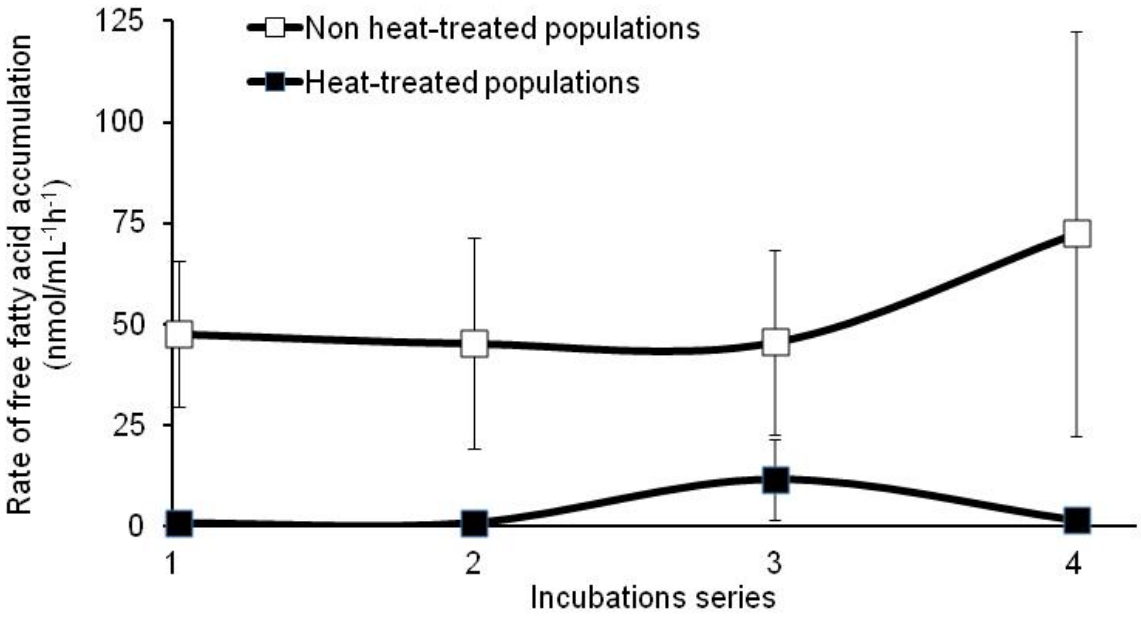

Figure 3.1 Mean (± SD) rates of free fatty acid accumulation during cultural adaptation of non- heat-treated (n = 6 per incubation series) and heat-treated ruminal microbes (n = 3 per incubation series) to an anaerobic (100% CO_2) basal rumen fluid/olive oil (5% vol/vol) medium via four consecutive, 48 h batch cultures at 39^{o}C. Main affect P-values from a general analysis of variance for heat treatment and incubation series was < 0.001 and 0.71, respectively, and 0.57 for the potential interaction.

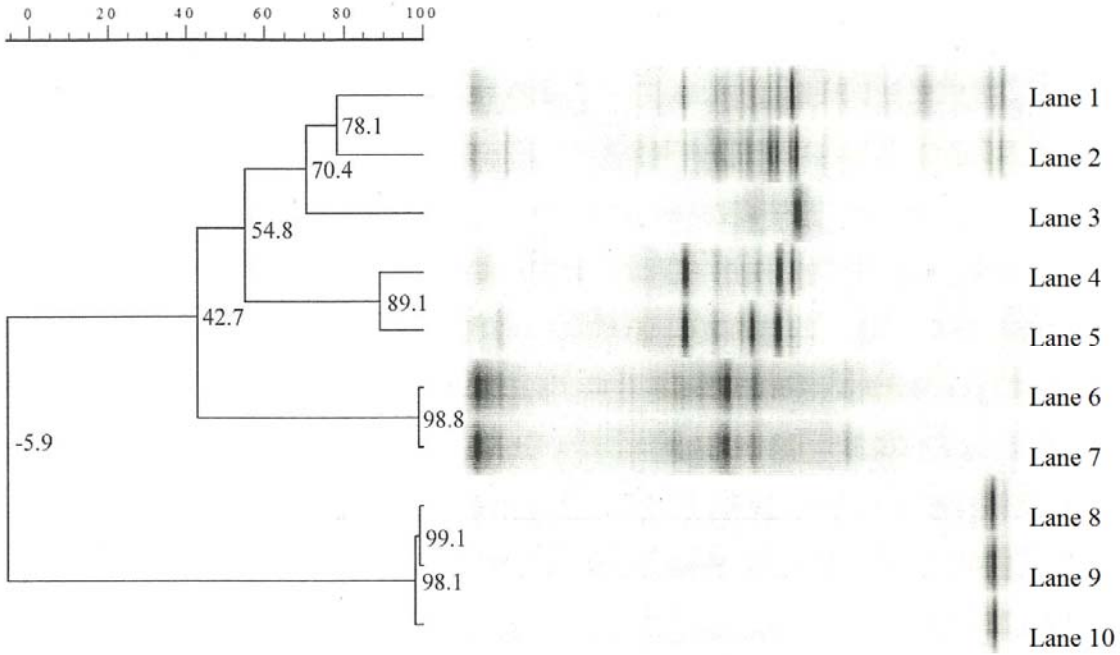

Figure 3.2 Denaturing gradient gel electrophoresis. Lanes 1 and 2; non-heat-treated populations adapted during four consecutive batch cultures in basal rumen fluid/olive oil medium and then likewise cultured a fifth time except in the absence (lane 1) or presence (lane 2) of added glycerol (6% vol/vol). Lane 3; positive control of *Clostridium sporogenes.* Lanes 4 and 5; heat-treated, populations adapted during four consecutive batch cultures in basal rumen fluid/olive oil medium and then likewise cultured a fifth time except in the absence (lane 4) or presence (lane 5) of added glycerol (6% vol/vol). Lanes 6 and 7; non-heat-treated and heat-treated rumen populations, respectively, sampled prior to consecutive batch culture. Lanes 8, 9, and 10 are pure cultures of *A. lipolyticus* 5s, *B. fibrisolvens* 49, and *P. acnes*.

control of *C. sporogenes* shared 55% similarity to heat treated samples (lane 3). Lanes 8, 9, and 10 represent pure cultures of *A. lipolyticus* 5s, *B. fibrisolvens* 49, and *P. acnes* and there was no similarity with lanes 4 and 5, which contained the heat-treated, adapted populations without and with glycerol supplementation.

Discussion

Lipase-expressing bacteria found in the rumen play a major role in changing the composition of lipids entering the rumen. (Polan et al., 1964). Isomerization requires a free carboxyl group (Kepler et al., 1970), thus establishing lipolysis as a prerequisite for biohydrogenation. There are several different types of microorganisms found in the rumen capable of hydrolyzing fatty acids from their TAG precursors. Because lipase activity by pure cultures of the prominent ruminal lipase-producing bacteria *A. lipolyticus* 5s, *B. fibrisolvens* 49, *P. acnes,* and *P. avidum* is insensitive to glycerol, it is apparent that other important glycerol-sensitive lipolytic bacteria have yet to be identified and characterized from the rumen. Several lipolytic clostridial species have been isolated from dilute rumen populations (van Gylswyk and van der Toorn, 1987; Jarvis et al., 1998; Krueger et al., 2008). However, there is little information regarding these species' contribution to overall ruminal lipolytic activity. The heat treatment used in the present study has been applied in a number of studies (heat application at 80°C for 10 minutes) to eliminate non-spore-forming bacteria during isolation, which allows for spore-forming bacteria, such as *Clostridium* species, to survive (Alexander, 1977; Doyle, 1989).

Trial 1 revealed a significant loss of lipolytic activity when ruminal populations were heat-treated prior to consecutive batch culture in the basal rumen fluid/olive oil medium. Heat-treated cultures had little to no activity during the first two incubations series. Rates of lipolysis never fully recovered even after the next two consecutive batch cultures, achieving at most only 25% of the activity expressed by the non-heat-treated populations (Figure 3.1). Our DGGE analysis, in Figure 3.2, of heat-treated populations sampled after their fifth and final incubation series (lanes 4 and 5) showed marked changes in cultural diversity, as evidenced by decreasing intensity of numerous bands and a marked increase in intensity of other bands, as compared to the adapted, non-heat-treated populations (lanes 1 and 2) or to the original rumen fluid populations (lane 6). Additionally, there were no band similarities between heat-treated populations (lanes 4 and 5) and bands of prominent, lipase producing bacteria *A. lipolyticus* 5s, *B. fibrisolvens* 49, and *P acnes* (lanes 8, 9, and 10) indicating their absence in the heat-treated population. This change in cultural diversity was not unexpected, as members of the population killed by heat treatment would not propagate and their cells would have been diluted upon each successive consecutive batch culture. Low recovery in lipolytic activity by heat-treated population suggests that the number of lipolytic bacteria was markedly reduced. Conversely, the modest increase in lipolytic activity during the final two incubation series likely reflects the germination of spores that survived heat treatment, which in the rumen environment would be presumptive of clostridial species. It is clear, however, that the heat-resistant population never fully recovered, likely because the medium, which contained olive oil as the major added energy substrate and

contained little carbohydrate, was insufficient to support the growth of the surviving population.

With respect to the non-heat-treated populations in both Trials 1 and 2, rates of FFA accumulation remained similar across the consecutive incubations or adaptation to the basal rumen fluid/olive oil medium. This suggests that, despite the marked changes in diversity, as evidenced by DGGE analysis of the adapted and original rumen fluid populations, lipolytic activity was maintained at or near the maximum sustainable levels during culture of these non- heat-treated populations in the basal rumen fluid/olive oil medium. Also, lipolytic activity of non-heat-treated populations in both Trials 1 and 2 were reduced by glycerol treatment. Similarly, our laboratory has demonstrated that glycerol supplementation inhibits lipolytic activity of mixed populations of ruminal microbes (Krueger et al., 2010; Edwards et al., 2012).

Results from our DGGE analysis of the cultures that were first heat-treated and then adapted to the basal rumen fluid/olive oil medium demonstrate an effect of glycerol treatment on cultural diversity. Increased intensification of one band and diminished intensification of another band in the glycerol-supplemented population were readily apparent. For the heat-treated populations in Trial 2, these bacteria were not subjected to DGGE diversity analysis because the heat treatment was applied to those populations after they were first adapted, and they thus had little opportunity to dilute dead and non-propagating cells during the glycerol test in the fifth and final culture series.

In conclusion, results from these studies confirm the sensitivity of mixed populations of ruminal microbes to glycerol supplementation. This sensitivity also was

shown to be expressed in populations that received the heat treatment both prior to and after culture adaptation. Early research suggested that spore-forming anaerobes were insignificant contributors to most metabolic processes in the rumen (Doetsch and Robinson, 1953). More recently, however, *Clostridium* species have been found to contribute significantly to the overall functionality of ruminal digestive activity. For instance, *C. lochheadii* has been found to contribute a considerable proportion of celluloytic activity (Hungate, 1957). Additionally, *C. proteoclasticum* was identified as a key bacteria in the biohydrogenation pathway producing stearic acid (Wallace et al., 2006), and later was renamed *Butyrivibrio proteoclasticus* (Moon et al., 2008).

Considering that the genus *Clostridium* is made up of highly diverse species it is not unreasonable to conclude that certain lipase-producing *Clostridium* species may contribute to the overall lipolytic activity in the rumen, and that these may include glycerol-insensitive and glycerol-sensitive species. Abo El-Nor et al. (2010) reported that DNA concentrations for *C. proteoclasticum* decreased during culture with glycerol supplementation. Additional pure culture research is warranted to further elucidate the main *Clostridium* species responsible for lipolytic activity in the rumen and discern which species are glycerol-insensitive and glycerol-sensitive. Advancement of our knowledge regarding the major bacterial contributors to lipolysis will aid in the development of cost effective ways to protect lipids from lipolysis as a way to prevent rumen biohydrogenation thereby reducing the saturated fatty acid content of ruminant-produced foods.

CHAPTER IV

IMMUNOGENIC INHIBITION OF PROMINENT RUMINAL BACTERIA AS A MEANS TO REDUCE LIPOLYSIS AND BIOHYDROGENATION ACTIVITY *IN VITRO*

Introduction

According to Resurreccion (2003), U.S. consumers find healthfulness to be the most important quality trait of beef. Unsaturated fatty acids have been shown to aid in several tissue metabolic processes, making their increased content in beef desirable from a health aspect. Food products derived from cattle, however, contain a high concentration of saturated fats relative to plants, a consequence of microbial lipolysis and biohydrogenation in the rumen. In order for biohydrogenation to occur, FFA must first be hydrolyzed from their TAG precursors (Harfoot and Hazlewood, 1997), a process known as lipolysis. Several microorganisms have been recognized as contributors to ruminal lipolysis and biohydrogenation activity. The present study focuses on the extracellular, lipase-producing bacteria *Anaerovibrio lipolyticus* 5s*, Butyrivibrio fibrisolvens* H17C*, Propionibacterium avidum,* and *P. acnes*.

Strains of *B. fibrisolvens* contribute to both lipolysis (Hazlewood and Dawson, 1979) and biohydrogenation (Polan et al., 1964; Wallace et al., 2006). *Butyrivibrio fibrisolvens* facilitates the initial isomerization reaction for both linoleic and α-linolenic acid in the biohydrogenation pathway (Polan et al., 1964). We hypothesized that by immunizing ruminants against bacteria and/or their purified enzymes that contribute to

lipolysis or the initial stages of biohydrogenation, saturated fatty acids would be significantly reduced in their meat and milk. This study examined antibodies created against these ruminal bacteria and their ability to inhibit lipolysis and, in the case of *B. fibrisolvens* H17C, biohydrogenation. We hypothesized that, because lipolysis is an extracellular process, the antibodies would be effective at inhibiting this process. Additionally, an antibody generated against a purified *Pseudomonas* lipase (Sigma Aldrich, Milwaukee, WI) was included in this study to determine if lipolytic activity could be inhibited to a greater degree by using an antibody with more specificity for the lipase itself compared to an antibody against whole cells.

Materials and Methods

Treatments

These experiments tested the hypothesis that the ruminal processes of lipolysis and biohydrogenation could be inhibited immunogenically. Egg yolk antibodies were created against the bacteria *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum,* and *P. acnes*. An antibody also was created against a purified *Pseudomonas* lipase. In the initial study, each antibody was tested individually at increasing doses (0, 0.025, 0.1, 0.3, 0.6 mL) ranging from 7-11 mg protein/mL against the bacteria they were created against in pure culture.

In a subsequent study the *B. fibrisolvens* H17C antibody was examined for its ability to inhibit biohydrogenation against itself in pure culture. Culture tubes contained 0 or 0.6 mL of antibody, 4 mL bacteria cultured to late log phase, 2 mL anaerobic buffer,

21 g glass beads and 3 mg linoleic or α-linolenic acid. Extent of biohydrogenation was determined by examining biohydrogenation products between tubes containing antibody and tubes without antibody at 0, 3, and 6 h.

Microbial Preparation and Handling Procedures

Pure cultures of *A. lipolyticus* 5s and *B. fibrisolvens* H17C were obtained from Dr. Jay Yankee, Agriculture-Agri Food Canada. Strains of *P. avidum* and *P. acnes* were previously isolated from the rumen of a pastured cow (Krueger et al., 2008). For long-term preservation of pure cultures, bacteria were stored in 20% anaerobic glycerol at -80ºC. Upon removal from storage, each bacterium was revived via two consecutive 24 to 48 h cultures in 10 mL standard anaerobic broth supplemented with 2% pre-sterilized olive oil. Tubes were incubated at 39°C and agitated horizontally at 35 rpm in an Innova™ Model 4000 Shaking Incubator (New Brunswick Scientific Co., Inc., Edison, NJ).

Antibody Preparation

Anaerovibrio lipolyticus 5s, *B. fibrisolvens* H17C, *P. avidum*, and *P. acnes* cells were harvested from 20-mL cultures and washed two consecutive times with 0.1 M sodium phosphate buffered saline (PBS), pH 7.0. Following the final wash, bacterial cells and the purified *Pseudomonas* lipase were suspended with 1 mL of PBS and 1 mL of Adjuvant Titer Gold Max (Sigma Aldrich, Milwaukee, WI). Three chickens were injected for each bacteria/lipase at 6 different locations at 3 wk intervals for 3 separate

injections. After the first injection, normal saline was used in place of the adjuvant. One week following the final injection, eggs were harvested and antibodies were extracted from yolks using Eggcellent IGY purification kit purchased from Pierce (Rockford, IL). Thermo Scientific Modified Lowry's Assays kit (Thermo Fisher Scientific, Waltham, MA) was used to determine antibody concentrations in the extracted material. Chickens were cared for according to procedures approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee (College Station, TX; protocol #2012004).

Standard Anaerobic Medium Formulation

Standard anaerobic broth medium contained (per liter): 292 mg each of K_2HPO_4 and $KHPO_4$, (JT Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ), 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_20$, 4,000 mg Na_2CO_3, 600 mg cysteine-HCl (J.T. Baker), 10 g trypticase (BBL Microbiology Systems, Cockeysville, MD), 2.5 g yeast extract (Difco; Becton, Dickinson and Company, Sparks, MD), branched-chain fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), 2 µg hemin, vitamin mix (20 mg each thiamine, pantothenate, nicotinamide, pryridoxine HCL, riboflavin, 1 mg *p*-aminobenzoic acid, 0.5 mg biotin, 0.5 mg folic acid, 0.2 mg vitamin B-12, and 0.5 mg lipoic acid), 0.1 g D-glucose and trace minerals (Cotta and Russell, 1982). All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Medium was boiled to displace dissolved oxygen and then cooled on ice while being saturated with a continuous flow of O_2-free CO_2 for 40

min. Media was then distributed (80-mL) to 100-mL glass walled vials using the anaerobic Hungate technique and capped (Bryant, 1972). Media was autoclaved immediately and cooled to room temperature before use.

Determination of Antibody Effectiveness at Reducing Lipolytic Activity against Pure Cultures of Ruminal Bacteria

Enzymatic assays were conducted in a 4 × 5 × 5 factorial arrangement to test anti-*A. lipolyticus* 5s, anti-*B. fibrisolvens* H17C, anti-*P. avidum,* anti-*P. acnes,* and anti-*Pseudomonas* lipase antibodies against the lipolytic activity of *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum, and P. acnes* at five different antibody doses (0, 0.025, 0.1, 0.3, and 0.6 mL) ranging from 7-11 mg protein/ml. Each revived bacterium was grown in 80-mL of standard anaerobic medium supplemented with 5% olive oil. Cells were obtained from cultures grown to late log phase and 4 mL was transferred anaerobically into assay tubes pre-loaded with 21-g glass beads (which served as a solid support matrix), 0.3-mL olive oil, and 2-mL anaerobic buffer, which was prepared similarly to the media but only contained minerals and cysteine-HCl. Each antibody dose was then separately administered to each bacterium. Assay tubes were incubated in the Innova™ Model 4000 Shaking Incubator at 39°C at 35 rpm; reactions were stopped at 12 h by uncapping and freezing the tubes. Total FFA were extracted and concentrations determined colorimetrically (Kwon and Rhee, 1986).

Determination of Anti- B. fibrisolvens *H17C Antibody against Biohydrogenation Activity of* B. fibrisolvens *H17C*

Enzymatic assays were conducted to specifically test the anti- *B. fibrisolvens* H17C antibody against the biohydrogenation activity of *B. fibrisolvens* H17C. *Butyrivibrio fibrisolvens* H17C was grown from revived cultures in 80 mL of standard anaerobic medium. Cells were then obtained from cultures grown to late log phase and 4 mL was transferred anaerobically into assay tubes pre-loaded with 21 g glass beads and 2 mL anaerobic buffer as described above. Tubes also contained either 6 mg of linoleic or α-linolenic acid and 0 or 0.6 mL of the antibody. All treatments then assayed for 1-h and 3-h in the Innova™ Model 4000 Shaking Incubator at 39°C at 35 rpm. Reactions were stopped at each time point by uncapping and freezing the tubes. To determine the extent of biohydrogenation, total lipid was extracted from tubes by the method of Folch et al. (1957). After methylation (Morrison and Smith, 1964), the fatty acid methyl esters (FAME) were analyzed using a Varian gas chromatograph by the method of Archibeque et al. (2005). Separation of FAME was accomplished on a fused silica capillary column CPSil88 [100.0 m × 0.25 mm (i.d.)]. Helium was used for the carrier gas. After 32 min at 180°C, oven temperature were increased at 20°C/min to 225°C and held for 13.75 min. Total run time was 48-min. Injector and detector temperatures were at 270 and 300°C, respectively. Biohydrogenation was calculated as the percentage disappearance from the original 6 mg of linoleic or α-linolenic acid added. The remaining percentage of linoleic or α-linolenic acid was then converted to nmol using the molecular weight of each fatty acid.

Statistical Analysis

Free fatty acid data for each antibody against each bacterium were analyzed using a one-way ANOVA (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a LSD separation of means when $P < 0.05$. The model included main effects of increasing antibody dose; $Y_{ik} = \mu + \text{antibody dose}_i + \varepsilon_{ik}$, where Y_{ik} is the dependent variable, μ is the overall mean, antibody dose is the fixed effect of the experimental treatments (i = 1 to 4), and ε_{ik} is the error term. A polynomial contrast appropriate for nonequally spaced treatments was performed to determine if increasing doses of antibody resulted in a linear or quadratic trend in percent reduction of lipolytic activity.

Differences in biohydrogenation were tested using a two-way general ANOVA with a LSD comparison of means ($P < 0.05$). The model included main effects of increasing incubation points, with or without antibody, and their interaction; $Y_{ijk} = \mu + \text{incubation}_i + \text{antibody}_j + (\text{incubation} \times \text{antibody})_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, incubation period and antibody treatments are the fixed effects of the experimental treatments (i = 1 to 4; j = 1 to 2), incubation × antibody is the interaction, and ε_{ijk} is the error term.

Results

Antibody Inhibition of Lipolytic Activity in Pure Cultures of Ruminal Bacteria

Each antibody was tested against each individual bacterium (*A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum*, and *P. acnes*) to determine the level of cross reactivity of each antibody (Table 4.1). Increasing levels of each antibody were examined to

determine the most effective concentration of antibody for reducing lipolytic activity of each bacterium.

Anti-*Pseudomonas* lipase antibody caused the greatest reduction in lipolytic activity, averaging 78% reduction at the highest dose across all four bacteria. Additionally, with the exception of *B. fibrisolvens* H17C, the anti-*Pseudomonas* lipase antibody caused a linear decrease ($P < 0.05$) in lipolytic activity for each of the bacterium with increasing concentration. Anti-*Pseudomonas* lipase antibody resulted in a treatment effect ($P < 0.05$) specifically against *P. acnes*. Anti-*P. avidum* antibody caused a linear decrease ($P < 0.05$) in lipolytic activity against all bacteria tested except *A. lipolyticus* 5s, with a treatment effect ($P < 0.05$) against *B. fibrisolvens* H17C and *P. acnes*. The anti-*A. lipolyticus* 5s and anti-*P. acnes* antibodies were not as broadly effective at reducing lipolytic activity as the other antibodies, although both exhibited a linear dose effect ($P < 0.05$) against *P. avidum*. The antibodies against *B. fibrisolvens* H17C caused a linear decrease ($P < 0.05$) in lipolytic activity for *P. acnes* with a treatment effect ($P < 0.05$) for *A. lipolyticus* 5s. Lipolytic activity decreased ($P < 0.05$) quadratically as the antibody dose of the anti- *B. fibrisolvens* H17C increased against *A. lipolyticus* 5s and the anti-*P. acnes* increased antibody against *P. acnes*.

Antibodies tested in this study had varying degrees of effectiveness at reducing the lipolytic activity of each of the bacteria, with the exception of the anti-*P. acnes* antibody, which appeared to be ineffective against *A. lipolyticus* 5s cultures. Only the anti-*B. fibrisolvens* H17C antibody was effective at highly reducing lipolytic activity against the bacterium it was created against, with an almost 75% decrease at the highest

Table 4.1 Effect of antibody dose on ruminal lipase producing bacteria, least square mean as a percent of relative reduction in lipolytic activity.

		% reduction in lipolytic activity Dose Level							
Bacterium	Antibody	0.025 mL	0.1 mL	0.3 mL	0.6 mL	SEM	Treatment	Linear	P-value Quadratic
A. lipolyticus 5s	*A. lipolyticus* 5s[1]	0.07	0.00	0.00	18.02	9.01	0.442	0.218	0.345
	P. acnes[1]	0.00	0.00	0.00	0.00	------	------	------	------
	P. avidum[2]	40.18	30.02	63.72	63.07	22.60	0.298	0.250	0.776
	B. fibrisolvens H17C[2]	60.52	8.39	50.70	53.31	11.77	0.057	0.844	0.025
	Pseudomonas lipase[2]	34.33	33.76	76.36	76.46	14.05	0.093	0.028	0.982
B. fibrisolvens H17C	*A. lipolyticus* 5s[1]	33.93	39.89	31.94	41.87	16.97	0.970	0.840	0.909
	P. acnes[1]	36.32	56.32	64.60	47.65	13.26	0.512	0.496	0.826
	P. avidum[2]	30.23[b]	50.68[b]	52.86[ab]	92.12[a]	12.58	0.046	0.010	0.476
	B. fibrisolvens H17C[2]	47.50	47.94	86.87	74.50	13.81	0.188	0.088	0.655
	Pseudomonas lipase[2]	66.64	54.14	77.52	96.03	14.63	0.294	0.127	0.320
P. avidum	*A. lipolyticus* 5s[1]	13.85[b]	41.88[a]	54.03[a]	63.18[a]	8.28	0.014	0.003	0.287
	P. acnes[1]	39.57[c]	7.51[bc]	52.47[ab]	69.66[a]	4.50	0.010	0.003	0.937
	P. avidum[2]	6.71	14.12	31.95	59.00	14.64	0.131	0.028	0.521
	B. fibrisolvens H17C[2]	21.54	21.33	20.59	48.69	14.98	0.507	0.263	0.372
	Pseudomonas lipase[2]	33.81	36.43	37.72	81.35	14.10	0.125	0.052	0.184
P. acnes	*A. lipolyticus* 5s[1]	7.47	15.36	14.59	26.49	9.88	0.615	0.239	0.844
	P. acnes[1]	14.61	2.64	0.00	38.67	8.91	0.054	0.119	0.022
	P. avidum[2]	0.56[b]	9.59[b]	23.72[ab]	50.63[a]	9.18	0.022	0.004	0.358
	B. fibrisolvens H17C[2]	20.22	16.63	26.10	48.01	9.70	0.095	0.036	0.173
	Pseudomonas lipase[2]	4.76[b]	7.10[b]	19.92[b]	55.90[a]	7.88	0.006	0.002	0.065

Treatments were performed in triplicate

[abc] Means within rows with unlike superscripts differ

[1]Control values for *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum,* and *P. acnes* are 961.67, 3,361.01, 15,771.42 and 2,902.23 µmol/mL

[2]Control values for *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum,* and *P. acnes* are 4,008.33, 2,616.08, 9,105.44 and 4,542.85 µmol/mL

dose.

Effectiveness of Anti-B. fibrisolvens *H17C Antibody against Biohydrogenation Activity of Pure Cultures of* B. fibrisolvens *H17C*

The effectiveness of the anti-*B. fibrisolvens* H17C antibody against itself in pure culture at reducing biohydrogenation of linoleic and α-linolenic acid is shown in Figure 4.1. Biohydrogenation of α-linolenic acid was depressed by antibody by 45% at the 1-h time point, compared to the controls (92 ± 11 versus 166 ± 66 nmol mL^{-1} for control and treatment, respectively; mean ± SEM). At the 3 h time point the antibody reduced biohydrogenation of α-linolenic acid by 22% (153 ± 40 versus 120 ± 25 nmol mL^{-1} for control and treatment, respectively). Biohydrogenation of linoleic acid was not affected by the anti- *B. fibrisolvens* H17C antibody.

Discussion

Lipase-expressing bacteria found in the rumen play a major role in the alteration of lipids that enter the rumen. Several technologies have been developed to protect lipids from biohydrogenation, such as the use of calcium salts or encapsulation of fat in a matrix of aldehyde-treated protein (Perfield II et al., 2004; de Veth et al., 2005; Harvatine and Allen, 2006; Jenkins and Bridges, 2007). These techniques have shown varying degrees of success, although these technologies have presented with some limitations. These limitations include the diversity of lipase-producers in the rumen and their adaptability to nutritional sources. The technology explored in the present study

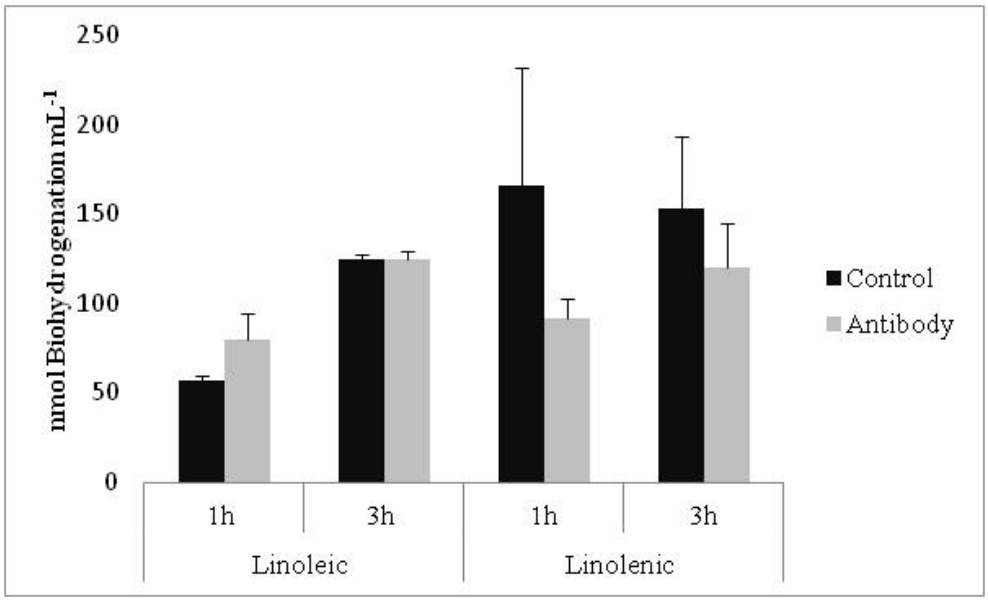

Figure 4.1 Biohydrogenation of linoleic or α-linolenic acid when incubated in pure culture of *B. fibrisolvens* H17C with and without anti-*B. fibrisolvens* H17C antibody. Bars depict means of 6 replications of experiments with error bars depicting 1 standard deviation from the mean.

focuses on protecting unsaturated fatty acids by preventing lipase-producing bacteria from creating a free carboxyl group, a requirement for biohydrogenation.

Extracellular lipases expressed by bacteria catalyze the hydrolysis of mono-, di- and triacylglycerols to FFA and glycerol (Pandey et al., 1999; Aravindan et al., 2007). Results from the present study indicated that antibodies created against specific rumen bacteria displayed prominent reduction in lipolytic activity for each bacterium in pure culture. This was expected due to the expression of extracellular lipases, which allows for direct interaction with the antibodies. There are several different types of microorganisms found in the rumen that are capable of lipolytic activity. This study focused on two organisms that have previously shown the greatest lipolytic activity *in vitro*, *P. acnes* and *P. avidum*. *Propionibacterium* are present in relatively high populations within the rumen. *Propionibacterium* population in the rumen have been established to be present between 10^4 and 10^6 CFU/mL of rumen fluid (Zimmer, 1999). *Butyrivibrio fibrisolvens* H17C and *A. lipolyticus* 5s also were investigated due to previous findings indicating them as major contributors to lipolysis (Polan et al., 1964; Henderson, 1971; Henderson and Hodgkiss, 1973; Prins et al., 1975).

Butyrivibrio species contribute mainly but not exclusively to the hydrolysis of galactolipids and phospholipids (Lourenço et al., 2010). *Anaerovibrio lipolyticus* has the ability to hydrolyze TAG and other esterified fatty acids (Henderson, 1971). *Anaerovibrio lipolyticus* is limited in its capacity to ferment carbohydrates, metabolizing only glycerol, fructose and ribose, which are fermented to acetic, propionic, and succinic acid (Henderson, 1975). Even though these bacteria are different, the antibodies created

against them caused reductions in lipolytic activity across each of the bacteria. This suggests that the lipase expressed by these bacteria may be genetically conserved. Therefore, any of these antibodies has potential to be effective against the majority of ruminal lipase-producing bacteria.

The use of the anti-*Pseudomonas* lipase in this study was designed to determine if using a purified lipase antibody would be more specific for inhibiting the lipase than using whole-cell antibodies. The anti-*Pseudomonas* antibody was the most effective at reducing lipolytic activity for all of the bacteria, averaging an almost 78% reduction in activity at the highest dose. These results show that the use of an antibody against a purified lipase may be more effective at reducing lipolysis then an antibody generated against whole-cells.

Unlike lipolysis, biohydrogenation takes place intracellularly (Chalupa and Kutches, 1968), and thus we hypothesized that the antibody would not be as effective at reducing biohydrogenation activity as lipolytic activity. However, the *B. fibrisolvens* H17C antibody reduced the hydrogenation of α-linolenic acid in pure cultures of *B. fibrisolvens* H17C. Maia et al. (2010) investigated the mechanisms by which PUFA affect the growth of *B. fibrisolvens* and found that linoleic and α-linolenic acid lengthened the lag phase of *B. fibrisolvens,* with α-linolenic acid having the greater effect. However, growth occurred only when PUFA had been converted to vaccenic acid. From these results, Maia et al. (2010) concluded that lipolysis and biohydrogenation occur to enable *B. fibrisolvens* to survive the bacteriostatic effects of PUFA. Results from the present study are consistent with these findings, in that more

biohydrogenation took place when incubations were performed with α-linolenic acid than with linoleic acid. Since the log phase is significantly shorter for *B. fibrisolvens* in the presence of linoleic acid, it is likely that the antibody did not have sufficient opportunity to disrupt the biohydrogenation process of *B. fibrisolvens* H17C.

In conclusion, these results indicate that antibodies against prominent ruminal bacteria contributing to lipolysis and biohydrogenation are effective at reducing the saturation of fatty acids. Additionally, each antibody displayed cross reactivity against other pure cultures of bacteria, suggesting genetic similarity between the lipases secreted by these bacteria. Therefore, each individual antibody may be effective against a variety of lipase-producing bacteria. Further research is warranted to determine if it is possible to immunize ruminants against these bacteria to increase the escape of unsaturated fatty acids from the rumen for absorption and assimilation.

CHAPTER V

IMMUNOGENIC INHIBITION OF MIXED CULTURE RUMINAL BACTERIA AS A MEANS TO REDUCE LIPOLYSIS AND FATTY ACID BIOHYDROGENATION *IN VITRO*

Introduction

Microbially mediated processes of lipolysis and biohydrogenation have a major impact on fatty acid composition of meat and milk from ruminants. Lipase-producing bacteria efficiently hydrolyze lipids, thereby liberating the free carboxyl group required for the initial step of biohydrogenation (Kepler et al., 1970). A high and regular inclusion of saturated fatty acids in human diets can contribute to several negative health effects, the most prominent being coronary heart disease (Grundy et al., 1988; Wahrburg, 2004).

This study examined an immunological approach to inhibiting bacterial contributors to both lipolysis and biohydrogenation in two separate experiments. For this study, antibodies were created against key ruminal bacteria involved in lipolysis and/or biohydrogenation and administered to mixed ruminal cultures individually to determine their effectiveness at inhibiting these processes. Antibodies generated were against the bacteria *Anaerovibrio lipolyticus* 5s, *Butyrivibrio fibrisolvens* H17C, *Propionibacterium avidum*, and *P. acnes*. The first study examined each of these antibodies separately and their ability to inhibit lipolytic activity in mixed culture. The subsequent study examined the *B. fibrisolvens* H17C antibody and its effectiveness at inhibiting biohydrogenation.

The initial step in the saturation of linoleic and α-linolenic acid involves the same isomerization reaction. *Butyrivibrio fibrisolvens* causes this initial reaction in both biohydrogenation pathways (Polan et al., 1964; Wallace et al., 2006). Therefore, two different oils were examined, linoleic acid-enriched corn oil and α-linolenic acid-enriched linseed oil. We hypothesized that, because lipase is extracellularly secreted by these bacteria, the antibodies would be effective at reducing lipolytic activity. We also predicted that the anti-*B. fibrisolvens* H17C antibody would have a more observable effect when used to protect linseed oil than corn oil, because linseed oil is unsaturated to a greater extent than corn oil.

Materials and Methods

Treatments

These experiments tested the hypothesis that the ruminal processes of lipolysis and biohydrogenation could be inhibited immunogenically. Egg yolk antibodies were created against the bacteria *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum,* and *P. acnes*. In the initial study each antibody was tested individually at increasing doses (0, 0.025, 0.1, 0.3, 0.6 mL) ranging from 7-11 mg protein/ml against ruminal mixed cultures.

In a subsequent study, the *B. fibrisolvens* H17C antibody was examined for its ability to affect biohydrogenation against mixed culture. Treatments were administered in a 2 × 2 × 3 factorial design and included 0 or 0.6 mL of antibody, 0.3 mL corn or linseed oil and three separate incubation lengths (0, 3, and 12 h).

Microbial Preparation and Handling Procedures

The mixed bacterial population used in this study was obtained from a cannulated cow grazing on predominantly rye grass pasture. Ruminal fluid was strained though a nylon paint strainer (Leyendecker et al., 2004) into a pre-warmed insulated container that had been flushed with warm water prior to sample collection. The container was filled completely (approximately 500 mL), capped and transported to the lab. Upon arrival at the laboratory, CO_2 was bubbled through the ruminal fluid to maintain anaerobiosis until use (within 30 min of collection). The cow was cared for according to procedures approved by Southern Plains Agricultural Research Center's Animal Care and Use Committee (Protocol #2012004).

Antibody Preparation

Anaerovibrio lipolyticus 5s, *B. fibrisolvens* H17C, *P. avidum*, and *P. acnes* cells were harvested from 20 mL cultures and washed two consecutive times with 0.1 M sodium phosphate buffered saline (PBS) at pH 7.0. Following the final wash, bacterial cells were suspended with 1 mL of PBS and 1 mL of Adjuvant Titer Gold Max (Sigma Aldrich, Milwaukee, WI). Three chickens were injected for each bacterium at 6 different locations at 3 wk intervals for 3 separate injections. After the first injection, normal saline was used in place of the adjuvant. One week following the final injection, eggs were harvested and antibodies were extracted from yolks using Eggcellent IGY purification kit purchased from Pierce (Rockford, IL). Protein content in the extracted antibody preparations was determined using a Thermo Scientific Modified Lowry

Protein Assay kit according to manufacturer's directions (Thermo Fisher Scientific, Waltham, MA). Chickens were cared for according to procedures approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee (College Station, TX; protocol #2010005)

Determination of Antibody Effectiveness at Reducing Lipolytic Activity in Mixed Rumen Cultures

Antibodies against *A. lipolyticus* 5s, *B. fibrisolvens* H17C, *P. avidum* and *P. acnes* were tested in a 4 × 5 factorial arrangement against ruminal mixed cultures. Each antibody was administered at five different antibody doses (0, 0.025, 0.1, 0.3, 0.6 mL) ranging from 7-11 mg protein/mL to determine their individual effectiveness at reducing the accumulation of FFA. Anaerobically, 6 mL of rumen fluid was transferred into tubes pre-loaded with each individual antibody at each dose, 21 g glass beads (which served as a solid support matrix), and 0.3 mL olive oil. Tubes were incubated in an Innova™ Model 4000 Shaking Incubator (New Brunswick Scientific Co., Inc., Edison, NJ) at 39°C at 35 rpm and reactions were stopped at 12 h by uncapping and freezing the tubes. Total FFA were extracted and concentrations determined colorimetrically (Kwon and Rhee, 1986). Lipolytic activity was calculated as percentage reduction or percent control of FFA accumulation relative to amounts produced in control cultures not containing antibody.

Determination of the Anti-B. fibrisolvens *H17C Antibody against Biohydrogenation Activity in Mixed Rumen Culture*

The anti-*B. fibrisolvens* H17C antibody was further tested for its ability to reduce biohydrogenation in ruminal mixed cultures. Under anaerobic conditions, 6 mL of mixed culture was transferred anaerobically into tubes pre-loaded with 0 or 0.6 mL antibody, 0.3 mL corn or linseed oil, and 21 g glass beads. Tubes were then incubated at 0, 3, or 12 h in the Innova™ Model 4000 Shaking Incubator at 39°C at 35 rpm. Reactions were stopped at each time point by uncapping and freezing the tubes.

Volatile fatty acids were measured by extracting 1 mL of incubated rumen fluid from each treatment and mixing it with phosphoric acid and pivalic acid solution (1.0% w/v) as an internal standard. The mixed solution was then centrifuged at 15,000 × *g* for 15 min and supernate was used to determine the concentration and composition of VFA using a gas chromatograph equipped with a flame ionization detector (FID) (Li et al., 2009).

Total lipid was extracted from tubes by the method of Folch et al. (1957). Accumulation of FFA were determined colorimetrically (Kwon and Rhee, 1986), biohydrogenation was determined by methylating the remaining lipid from samples (Morrison and Smith, 1964). The fatty acid methyl esters (FAME) then analyzed using a Varian gas chromatograph by the method of Archibeque et al. (2005). Separation of FAME was accomplished on a fused silica capillary column CPSil88 [100.0 m × 0.25 mm (i.d.)]. Helium was used for the carrier gas. After 32 min at 180°C, oven

temperature were increased at 20°C/min to 225°C and held for 13.75 min. Total run time was 48 min. Injector and detector temperatures were at 270 and 300°C, respectively.

Statistical Analysis

FFA data for each antibody in mixed culture were analyzed using a two-way ANOVA (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a LSD separation of means when $P < 0.05$. The model included main effects of antibody source, increasing dose of antibody and their interaction; $Y_{ijk} = \mu + \text{antibody}_i + \text{dose}_j + (\text{antibody} \times \text{dose})_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, antibody and dose are the fixed effects of the experimental treatments (i = 1 to 4; j = 1 to 5), antibody × dose is the interaction, and ε_{ijk} is the error term.

Differences in biohydrogenation and VFA were tested using a two-way general ANOVA with a LSD separation of means ($P < 0.05$). The model included main effects of increasing incubation points, with or without antibody, and their interaction; $Y_{ijk} = \mu + \text{incubation}_i + \text{antibody}_j + (\text{incubation} \times \text{antibody})_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, incubation period and antibody treatments are the fixed effects of the experimental treatments (i = 1 to 4; j = 1 to 2), incubation x antibody is the interaction, and ε_{ijk} is the error term.

Results

Effectiveness of Antibodies at Reducing Lipolytic Activity in Mixed Cultures

The effectiveness of each antibody at reducing lipolytic activity in ruminal mixed cultures, represented as a percent inhibition and percent control in FFA accumulation from that produced by controls cultures incubated without any antibody is show in Table 5.1. Total accumulation of FFA was measured as an index of lipolytic activity. The percent inhibition was less ($P < 0.05$) for *P. avidum* than for *A. lipolyticus*, *B. fibrisolvens*, and *P. acnes.* Similarly, the percent control was greater ($P < 0.05$) for *P. avidum* and *B. fibrisolvens* than for *A. lipolyticus*, while the percent control for *P. acnes* was intermediate. The percent control for anti-*A. lipolyticus* 5s, anti-*B. fibrisolvens* H17C, anti-*P. acnes,* and anti-*P. avidum* antibodies was less ($P < 0.05$) for all doses than for control. Percent inhibition and percent of control in FFA accumulation did not differ ($P > 0.05$) among each dose for any of the antibodies

*Effect of Anti-***B. fibrisolvens** *H17C Antibody on Lipolysis when Administered to Mixed Culture Supplemented with Two Different Oil Substrates*

Corn and linseed oil were used to further examine the ability of the antibody created against *B. fibrisolvens* H17C to reduce lipolytic activity. Accumulation of FFA for both corn and linseed oil, at the three incubation points, with and without the inclusion of the anti-*B. fibrisolvens* H17C antibody, is shown in Figure 5.1. Cultures incubated with added linseed oil treated without antibody displayed an increase from 4.3 to 35.0 μmol of FFA from the 0 to 3 h incubations. The antibody-treated cultures exhibited a 2.8 to

Table 5.1 Percent inhibition and percent of control of antibody source and dose on FFA release in ruminal mixed culture.

Antibody, *n*	Treatment (mL)	Percent Inhibition	Percent Control
Control[1], *n* = 12	0	---	100
A. lipolyticus, *n* = 3			
	0.025	31.53	68.47
	0.10	18.99	81.01
	0.30	35.00	66.01
	0.60	34.50	65.50
B. fibrisolvens, *n* = 3			
	0.025	25.03	74.97
	0.10	7.62	92.38
	0.30	16.35	83.65
	0.60	29.29	70.71
P. acnes, *n = 3*			
	0.025	22.78	77.23
	0.10	30.31	69.69
	0.30	9.99	90.01
	0.60	15.69	84.31
P. avidum, *n* = 3			
	0.025	3.47	96.53
	0.10	10.71	89.29
	0.30	10.93	89.07
	0.60	7.23	92.77
	SEM	2.10	2.24
P-values	Antibody	0.002[2]	0.012[3]
	Dose	0.757	0.002[4]
	Antibody × Dose	0.244	0.089

[1]Control value 9.48 μmol/mL
[2]The percent inhibition was less for *P. avidum* than for *A. lipolyticus*, *B. fibrisolvens*, and *P. acnes.*
[3]The percent control was greater for *P. avidum* and *B. fibrisolvens* than for *A. lipolyticus*. *P. acnes* was intermediate.
[4]The percent control was less for all doses than for control; there was no difference in percent control among doses.

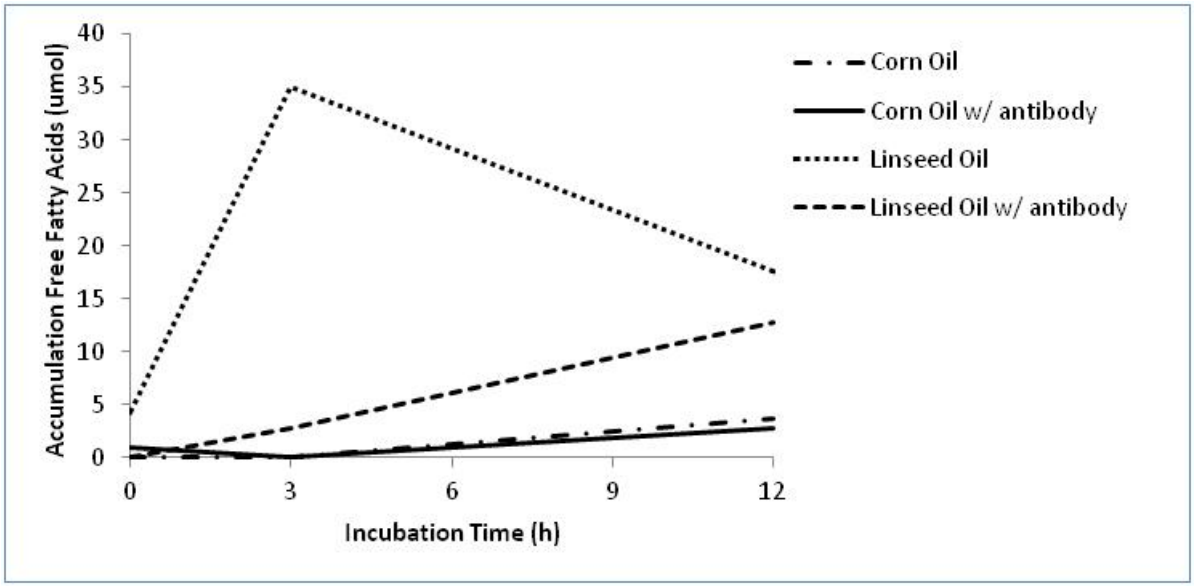

Figure 5.1 Total accumulation of FFA (µmol) from corn oil or linseed oil as a measure of lipolytic activity by mixed rumen culture with or without the treatment of 0.6 mL anti- *B. fibrisolvens* H17C antibody at increasing incubation times. Treatments were performed in triplicate.

12.8 μmol increase in FFA over the 3-h incubation period. At 3-h to 12-h, there was a decrease in total fatty acid accumulation for the non antibody-treated cultures containing linseed oil. Samples with linseed oil plus antibody had very little FFA accumulation until the 12-h incubation. Treatments in which corn oil served as the substrate displayed little to no FFA accumulation across all incubation points, independent of the presence of the antibody.

Biohydrogenation Products of Linseed Oil at Increasing Incubation Times with and without the Anti- B. fibrisolvens *H17C Antibody*

The fatty acid composition of the linseed oil-supplemented incubations at each of the three incubation points, with and without antibody is shown in Table 5.2. Increased incubation time affected ($P < 0.05$) all fatty acids, with or without antibody. Palmitic (16:0), stearic (18:0), and oleic acid (18:1n-9) increased ($P > 0.05$), while linoleic (18:2n-6), α-linolenic acid (18:3n-3), and *trans*-biohydrogenation products decreased ($P < 0.05$). Antibody treatment did not affect ($P > 0.05$) fatty acid composition in incubations supplemented with linseed oil. However, there was a tendency ($P = 0.056$) for an incubation × antibody interaction for the accumulation of *trans*-biohydrogenation products. At the 12 h incubation period, there were less *trans*-products produced when the antibody was present than when it was absent.

Volatile fatty acid compositions measured in the incubations supplemented with linseed oil and with or without antibody are presented in Table 5.3. Acetate decreased ($P < 0.05$) with increasing incubation time. *Iso*-butyrate, butyrate, *iso*-valerate, and valerate

Table 5.2 Effect of incubation time and antibody on fatty acids of mixed cultures in the presence of linseed oil; least square means

	μmol fatty acid									
Incubation	0 h		3 h		12 h				*P*-value	
Fatty Acid	Control	Antibody	Control	Antibody	Control	Antibody	SEM	Time	Antibody	T × A
16:0	10.28	10.08	10.53	10.90	11.11	11.48	0.25	0.003	0.379	0.428
18:0	7.94	7.34	7.50	8.40	9.32	9.07	0.32	0.001	0.954	0.093
18:1	28.58	28.53	28.67	28.63	29.62	29.62	0.2	0.001	0.865	0.986
18:2	21.09	21.30	20.97	20.71	18.39	18.57	0.15	<0.001	0.740	0.263
18:3	74.34	75.64	74.89	73.30	57.54	60.05	0.9	<0.001	0.332	0.105
Trans-fatty acids[1]	0.73	0.10	0.17	1.03	17.03	14.20	0.69	<0.001	0.148	0.057

Treatments were performed in triplicate

[abcd] Least square means within rows with unlike superscripts differ ($P < 0.05$)

[1]*Trans*-fatty acids equal sum of 18:1 *trans*-11, 18:2 *trans, cis*, and 18:2 *cis, trans*

Table 5.3 Effect of antibody and incubation time on volatile fatty acid accumulation in mixed culture in the presence of linseed oil; least square means

	mmol/100 mmol									
Incubation	0 h		3 h		12 h				*P*-value	
Fatty Acid	Control	Antibody	Control	Antibody	Control	Antibody	SEM	Time	Antibody	T × A
Acetate	64.07	65.47	62.30	61.84	60.40	59.89	0.89	0.001	0.852	0.497
Propionate	16.62	17.14	17.30	17.23	16.73	16.83	0.27	0.200	0.409	0.538
Iso-Butyrate	1.18^{d}	1.213^{d}	1.34^{cd}	1.73^{a}	1.52^{bc}	1.69^{ab}	0.06	<0.001	0.003	0.048
Butyrate	11.00	10.95	12.59	12.04	14.58	13.36	0.32	<0.001	0.041	0.229
Iso-Valerate	1.60^{b}	1.35^{b}	1.67^{b}	2.22^{a}	2.31^{a}	2.44^{a}	0.11	<0.001	0.126	0.010
Valerate	1.70^{bcd}	1.07^{d}	1.34^{cd}	1.78^{abc}	2.06^{ab}	2.39^{a}	0.21	0.004	0.785	0.044

^{abcde} Least square means within rows with unlike superscripts differ ($P < 0.05$)

increased ($P < 0.05$) with increasing time of incubation. *Iso*-butyrate increased ($P < 0.05$) with antibody treatment while butyrate decreased ($P < 0.05$) with antibody treatment. The VFA *iso*-butyrate, *iso*-valerate, and valerate displayed an incubation × antibody interaction. The VFA were increased by antibody treatment at 3 h, but not at 12 h of incubation.

Biohydrogenation Products of Corn Oil at Increasing Incubation Times with and without the Anti-B. fibrisolvens *H17C Antibody*

There was little to no accumulation of FFA in incubations supplemented with corn oil over the 12-h incubation period and consequently, biohydrogenation was not observed (Figure 5.1). Palmitic, oleic, and linoleic acid did not change over time with or without antibody treatment. *Trans*-biohydrogenation products also were not detected at any time point with or without antibody treatment.

Concentrations of *iso*-butyrate, *iso*-valerate, and valerate increased ($P < 0.05$) over incubation periods (Table 5.4). Antibody caused an increase ($P < 0.05$) in acetate. A significant incubation × antibody interaction was observed for accumulations of acetate, propionate, butyrate, and *iso*-valerate. Acetate was increased by antibody treatment at 0 h, but not at the other incubations. Propionate was increased by antibody treatment at 0 and 3 h but not at the 12 h incubation. Butyrate was increased by antibody treatment at the 3 h incubation but decreased at the 12 h incubation. *Iso*-valerate was decreased by antibody treatment only at the 0 h incubation.

Table 5.4 Effect of antibody dose and incubation time on mixed culture in the presence of corn oil, least square means of volatile fatty acid products

	mmol/100 mmol									
Incubation	0 h		3 h		12 h				*P*-value	
Fatty Acids	Control	Antibody	Control	Antibody	Control	Antibody	SEM	Time	Antibody	T × A
Acetate	47.29[b]	53.30[a]	49.16[b]	49.24[b]	48.06[b]	48.41[b]	1.00	0.162	0.022	0.019
Propionate	26.16[ab]	26.92[a]	26.06[ab]	27.25[a]	27.64[a]	25.00[b]	0.58	0.848	0.637	0.012
Iso-Butyrate	0.93	0.48	0.63	0.68	1.12	1.10	0.16	0.026	0.295	0.270
Butyrate	14.50[a]	14.14[ab]	13.32[bc]	14.37[a]	14.72[a]	12.45[c]	0.30	0.084	0.054	0.001
Iso-Valerate	1.34[a]	0.21[b]	0.72[b]	0.67[b]	1.43[a]	1.92[a]	0.19	0.004	0.165	0.004
Valerate	5.78	4.54	5.49	5.64	6.41	6.03	0.32	0.020	0.088	0.137

[abcd] Least square means within rows with unlike superscripts differ ($P < 0.05$)

Discussion

Bacteria examined in this study produce extracellular lipases/esterases, making them contributors to the saturation of fatty acids in the rumen. *Anaerovibrio lipolyticus* 5s produces two lipases, an esterase associated mainly within the cell, and a lipase which is secreted (Henderson and Hodgkiss, 1973). Additionally, *P. avidum* and *P. acnes* produce a number of extracellular enzymes, one of which is a lipase (Kellum et al., 1970; Hassing, 1971; Greenman et al., 1981; Ingham et al., 1981). Unlike the extracellular lipase producers in this study, *B. fibrisolvens* produces an extracellular esterase instead of a lipase; however, it displays the properties of a lipase. *Anaerovibrio lipolyticus* 5s showed the lowest percent of control in lipolytic activity from the controls. While *A. lipolyticus, B. fibrisolvens*, and *P. acnes* showed the highest percent inhibition from controls. The anti-*P. avidum* antibody was the least effective at reducing lipolytic activity, but antibody reduced the accumulation of FFA no matter the dose. The non-significant dose effect indicates that across the doses tested in this project a lesser antibody dose is just as effective as a greater dose. Because the lipases/esterases of the bacteria examined in this study are produced extracellularly and not protected by cell membranes these reductions in lipolysis are expected due to the antibody having optimal interaction with the lipase/esterase. We conclude that, since the process of lipolysis occurs extracellular, an antibody created against a purified lipase may have potential to be more effective than the antibodies against whole cells, such as the preparation used in this study.

In addition to *B. fibrisolvens* contributing considerably to ruminal lipolytic activity (Hazlewood and Dawson, 1979), it also facilitates the initial isomerization step of the biohydrogenation process of fatty acids linoleic and α-linolenic acid (Polan et al., 1964; Wallace et al., 2006). Thus, the anti-*B. fibrisolvens* H17C antibody was further examined in mixed culture supplemented with either corn oil enriched with linoleic acid or linseed oil, enriched α-linolenic acid, at different points during incubation. The antibody was highly effective at reducing the accumulation of FFA in incubations containing linseed oil in mixed cultures, as predicted from cultures incubated with olive oil. The non antibody-treated tubes containing linseed oil showed considerable accumulation of FFA. Most bacteria involved in the hydrolysis of TAG and phospholipids can utilize the free glycerol arising from this process. *Anaerovibrio lipolyticus (Prins et al., 1975)* and both *P. avidum* and *P. acnes* (Stjernholm and Wood, 1960; Holdeman et al., 1977; Stackebrandt and Schaal, 2006; Jarvis and Moore, 2010) are all capable of fermenting glycerol. However, *B. fibrisolvens* is incapable of fermenting glycerol (Bryant and Small, 1956). Thus, *B. fibrisolvens* likely expresses lipolytic activity to acquire long chain fatty acids for nutritional sources and their cell membranes (Hazlewood et al., 1980). Choi and Song (2005) examined the effects of 18-carbon PUFA on direct incorporation into rumen bacteria by adding 60 mg of linoleic acid or α-linolenic acid into rumen fluid, mixed culture solutions. The amount of fatty acids incorporated into the bacteria following a 12-h incubation were 1.20 mg and 0.43 mg/30 mL rumen fluid for linoleic and α-linolenic acid, respectively (Choi and Song,

2005). The incorporation of fatty acids back into the cell membrane may explain the decrease in the accumulation of FFA observed between the 3- and 12-h time points.

The results for cultures incubated in the presence of corn oil showed little to no accumulation of FFA whether the antibody was present or not. This suggests that the fatty acid composition of corn oil may exhibit protective effects against lipolytic activity. This is consistent with our previous findings (Edwards et al., 2013), which showed that corn oil displayed considerable resistance to ruminal lipolysis. Corn oil contains 29% oleic acid, 57% linoleic acid, and 1% α-linolenic acid, with the unsaturated fatty acids concentrated at the *sn*-2 position. Flaxseed oil contains 16% oleic acid, 17% linoleic acid, and 57% α-linolenic acid. Linoleic acid is concentrated in the *sn*-2 position, whereas α-linolenic acid is more concentrated at the *sn*-2 and *sn*-3 positions (60% of fatty acids at those positions) than at the *sn*-1 position (53% of *sn*-1 fatty acids). Results from the present study suggest that either the fatty acid composition or the *sn*-position distributions of the substrate oils influenced their susceptibility to hydrolysis by these cultures of ruminal bacteria.

In order for biohydrogenation to occur, fatty acids must first be hydrolyzed from their TAG precursors (Harfoot and Hazlewood, 1997). This coincides with our results showing that the majority of FFA accumulation took place between the 0- to 3-h incubations and biohydrogenation subsequently followed during the 3- to 12-h incubations. The fatty acid composition of linseed oil after incubation indicates that fatty acids linoleic and α-linolenic acid were hydrogenated to oleic, stearic, and palmitic acid. Also, less *trans*-fatty acids were produced at the longer incubation time when the

antibody was present. The presence of fewer *trans*-fatty acids, a major index of biohydrogenation, indicates that the antibody disrupted biohydrogenation in addition to lipolysis. There also was an increase in the branched- and mid- chain VFA when the antibody was present over time compared to when it was absent in both the linseed oil and corn oil incubations. This increase in branched-chain VFA further demonstrates that the antibody was causing a change in the activity of the bacterial populations. Because lipolysis is a prerequisite to biohydrogenation, the fatty acid composition remained unchanged in cultures incubated in the presence of corn oil.

In conclusion, each antibody produced against lipase/esterase-producing bacteria was effective at reducing the accumulation of FFA by mixed ruminal cultures. Additionally, the anti-*B. fibrisolvens* H17C antibody displayed protective effects from the biohydrogenation of fatty acids in mixed culture. However, the slight increase in the accumulation of FFA, when the antibody was incubated with linseed oil between the 3 and 12 h incubations, suggest that the antibody may be subject to fermentative degradation in the rumen. Thus, for application purposes, *in vivo* the antibody would need to be introduced in a manner where it would be replenished continually. This study suggests that it may be possible to significantly reduce the saturation of fatty acids by immunizing ruminants against bacteria that contribute to ruminal lipolysis and biohydrogenation.

CHAPTER VI

CONCLUSIONS

Dietary lipids are readily hydrolyzed and the resulting fatty acids are extensively saturated by microflora uniquely found in ruminal animals. As a result, only a small proportion of MUFA and PUFA are absorbed and assimilated into the meat and milk of ruminants. There have been several strategies developed to protect lipids from ruminal lipolysis. These strategies include glycerol supplementation (Krueger et al., 2010; Edwards et al., 2012), formaldehyde-protected fatty acids (Faichney et al., 1973; Garrett et al., 1976; Hogan and Hogan, 1976), feeding calcium salts (de Veth et al., 2005; Harvatine and Allen, 2006), and amide-protected fatty acids (Perfield II et al., 2004). Additionally, feeding diets that are supplemented with oils such as fish (Chow et al., 2004; Wasowska et al., 2006), linseed (Morris, 2008a), sunflower (Sackmann et al., 2003), and vegetable oil (Rego et al., 2005) have been examined in hopes that their high concentrations of MUFA and PUFA would allow for a greater escape of unsaturated fatty acids from the rumen. These approaches have all shown merit but also have met with a number of limitations, such as the diversity of lipase-producers in the rumen and the adaptability of these microbes. The goal of this research was to characterize prominent ruminal bacteria responsible for the saturation of fatty acids in the rumen. This was followed by the development of an approach that effectively promoted ruminal escape and intestinal absorption of unsaturated fatty acids.

In the initial experiment, glycerol and glucose were used to characterize lipase-producing bacteria that are currently established as prominent contributors to ruminal lipolytic activity: *A. lipolyticus* 5s, *B. fibrisolvens* 49, *P. avidum, and P. acnes*. Glycerol and glucose were used for characterization in this study because of their known opposing effects on lipolysis in mixed culture. As expected glycerol decreased the accumulation of FFA while glucose increased FFA accumulation of mixed culture. The opposite was observed for pure cultures showing an increase in FFA accumulation when incubated with glycerol and a decrease when incubated with glucose. The only exception being *P. avidum*, which similar to mixed cu0lture, displayed an increase in FFA accumulation when glucose was present. Thus, results from this study indicated that the lipase activity of *P. avidum* in the presence of glucose probably contributes more to lipolysis of dietary lipids in ruminants than *A. lipolyticus* 5s, *B. fibrisolvens* 49, or *P. acnes.* However, unlike mixed cultures, *P. avidum* was unaffected by glycerol, indicating that there may be an important glycerol-susceptible bacterial species contributing to ruminal lipolysis that has yet to be identified.

Clostridium species from rumen and non-rumen environments are capable of producing lipase. Because of the vast diversity of *Clostridium* species in the rumen an additional characterization study was performed examining rumen fluid that was enriched for presumptive *Clostridium* through heat treatment and its susceptibility to glycerol. Non-heat-treated ruminal mixed cultures were compared to heat-treated rumen cultures that were first heat-treated and then enriched in olive oil, or enriched and then heat treated. All populations were then supplemented with glycerol. Results from this

study showed reductions in FFA accumulation with glycerol treatment for non-heat-treated populations and heat-treated populations that were adapted to olive oil medium either before or after heat treatment. These results further confirm the sensitivity of mixed populations of ruminal microbes to glycerol supplementation and indicate that this sensitivity is expressed at least in part by heat-resistant bacterial populations, presumptively spore-forming *Clostridium* species. Further research is required to determine the role of *Clostridium* species in overall ruminal lipolysis and which species are glycerol-sensitive.

Until further research is performed to discern *Clostridium* species role in the rumen, *A. lipolyticus, B. fibrisolvens, P. avidum,* and *P. acnes* remain the primary focus of this research. The final experiments of this study were to develop a method to inhibit the saturation of fatty acids in the rumen. Antibodies were generated against these four bacteria and tested as a means to inhibit lipolytic activity. Additionally, an antibody generated against a *Pseudomonas* lipase also was developed and tested to determine if an antibody raised against a purified protein would be more effective than antibodies raised against whole cell preparations. We demonstrated that each individual antibody was effective in reducing the lipolytic activity of the respective species in pure culture as well as other pure cultures of bacteria. Reductions in lipolytic activity by all pure cultures of bacteria, in the presence of each antibody, suggest genetic similarity between the lipases. Therefore, each individual antibody may be effective against a variety of lipase-producing bacteria, which may include *Clostridium* species. Furthermore, the anti-*Pseudomonas* lipase showed the greatest reduction in lipolytic activity among the

pure cultures of bacteria. This is likely a result of the antibody having greater specificity for the lipase itself, indicating that the use of an antibody against a purified lipase is more effective at reducing lipolytic activity than against whole cell.

Because *B. fibrisolvens* H17C participates in the initial step of biohydrogenation of both linoleic and α-linolenic both free fatty acids were included in this study. In addition to disrupting the extracellular process of lipolysis, the *B. fibrisolvens* H17C antibody marginally reduced the hydrogenation of α-linolenic acid in pure cultures of *B. fibrisolvens* H17C, but not linoleic acid. This demonstrates that the intracellular process of biohydrogenation can also be reduced by use of an antibody that is produced against a bacterium involved in this process.

In a subsequent study, the four whole cell antibodies were further tested against mixed cultures. Similar to the results from the previous study examining pure cultures, results from this study showed that each antibody was effective at reducing the accumulation of FFA by mixed ruminal cultures. Additionally, the anti- *B. fibrisolvens* H17C was again tested in this study for its ability to inhibit biohydrogenation of mixed culture. Linoleic acid-rich corn and α-linolenic acid-rich linseed oil were used as the source of fatty acids. Corn oil exhibited resistance against lipolysis, independent of the antibody. Because lipolysis is a prerequisite for biohydrogenation, there was no biohydrogenation activity observed for the corn oil treatment. However, as with the initial study, the presence of the antibody displayed protective effects from the biohydrogenation of linseed oil by mixed culture as indicated by the accumulation of fewer *trans*-fatty acids with the antibody treatment. The overall conclusions of these

results indicate that it may be possible to immunize ruminant-species against bacteria that contribute to the lipolysis and biohydrogenation processes. Thus, would allow for increased escape of unsaturated fatty acids from the rumen for absorption and assimilation, resulting in the production of a ruminant product enriched with unsaturated fatty acids.

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