DEVELOPMENT OF METHODOLOGY AND CHARACTERIZATION OF RUMINAL LIPASE-PRODUCING BACTERIA *IN VITRO*

A Thesis

by

HOLLY DANIELLE EDWARDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2011

Major Subject: Animal Science

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Approved by:

Co-Chairs of Committee,	Rhonda K. Miller
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ABSTRACT

Development of Methodology and Characterization of Ruminal Lipase-Producing Bacteria *In Vitro*. (May 2011)

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Co-Chairs of Advisory Committee: Dr. Rhonda K. Miller
Dr. T. Matthew Taylor

Hydrolysis of dietary lipids to free fatty acids (FFA) is a prerequisite for ruminal biohydrogenation, a bacterially mediated process that extensively saturates unsaturated FFAs thus limiting the absorption and ultimate assimilation of these healthy nutrients into ruminant produced foods. Three experiments were conducted to learn how to better enrich, isolate and study lipolytic bacteria from the rumen while providing further characterization of four prominent lipase-producing bacteria that are known to be major contributors of lipolysis in the rumen. In experiment one the effects of various physical treatments on ruminal lipase activity were investigated by comparing incubation positions, glass bead levels, transfer techniques and combinations of headspace gasses. Based on results from this experiment an incubation system was established as a standard for subsequent studies for culturing and transferring mixed and pure cultures of ruminal bacteria. In experiment two the effect of glycerol on lipolysis by *Anaerovibrio lipolyticus 5S*, *Butyrivibrio fibrisolvens 49*, *Propionibacterium avidum,* and *Propionibacterium acnes* was examined. Two levels of glycerol were examined on lipase activity and results showed that glycerol inhibited rates of FFA accumulation at

both levels. In addition the mechanism behind glycerol inhibition was also examined by culturing and assaying activity of the four bacteria to determine if glycerol inhibition is a result of equilibrium displacement or lipase gene expression inhibition. Results indicated that higher and constitutively expressed lipase activity of *A. lipolyticus 5S* and *P. avidum* probably contribute more to lipolysis in ruminants than *P. acnes* and *B. fibrisolvens 49.* In the case of *P. acnes* and *B. fibrisolvens 49* cells, results suggest that lipase gene expression is down-regulated in these bacteria. Experiment three was conducted to further characterize the lipase activity of the four different bacteria by growing them with four different energy substrates and measuring enzyme activity at early logarithmic and stationary phase. Results from this study showed that diets containing a high content of oleic acid and linolenic acid promoted higher rates of lipolysis in the rumen. In accordance with findings in experiment two these results support that *P. avidum* may contribute to a higher amount of lipolysis than previously considered.

DEDICATION

To my parents, Tyler and Marcy Edwards, for their blind faith, support, love, and encouragement no matter the distance

ACKNOWLEDGEMENTS

I want to express my gratitude to Dr. Margaret Hardin and Dr. Rhonda Miller for giving me the opportunity to pursue a degree that appeals to my interest and for their constant support throughout. I also wish to thank Dr. T. Matthew Taylor for his time and guidance through the progression of my degree.

I would also like to extend a special thank you to Dr. Robin Anderson for his encouragement and direction during my masters program and for his dedication in helping me advance my research and knowledge.

I would also like to express my gratitude to Dr. Nathan Krueger for his technical assistance during the course of this study. I would also like to thank Seth Taylor for his assistance in the laboratory and during field activities.

Finally, I would like to express my appreciation to the College of Agriculture and Life Science and Southwest Agriculture Research Center for the allowing me the use of their facilities.

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CHAPTER I

INTRODUCTION

The consumption of ruminal products has been associated with causing a number of undesirable health effects. The high content of saturated and *trans* fats in the meat and milk derived from ruminant animals is the driving factor behind these health concerns. Therefore, ruminal producers are required to feed a low fat diet in an attempt to control the high levels of saturated fats.

Saturated fats accumulate in ruminant products as a result of the processes of lipolysis and biohydrogenation. Lipase-producing bacteria that are found in the rumen are responsible for freeing unsaturated fatty acids from a glycerol backbone, thereby allowing the double bond to be saturated. Eighty percent of dietary unsaturated fats that enter the rumen are effectively saturated. There are several bacteria in the rumen that contribute to lipolysis. These include *Anaerovibrio lipolyticus 5S, Butyrivibrio fibrisolvens 49, Propionibacterium avidum* and *Propionibacterium acnes.* Strategies that protect lipids from rumen lipolysis may effectively promote ruminal escape and intestinal absorption of unsaturated fatty acids thereby resulting in the production of value-added ruminant products enriched with healthy unsaturated fats.

The objectives of this study were; (1) to develop a forage free incubation and transfer system for culturing and handling lipase-producing bacteria, (2) to determine the individual responses of *A. lipolyticus 5S, B. fibrisolvens 49, P. avidum,*

This thesis follows the style of *Journal of Animal Science.*

and *P. acnes* when saturated with glycerol and characterize the mechanism of action of glycerol on ruminal lipase. It was hypothesized that introducing glycerol into bovine finishing diets at 5-20% would inhibit ruminal lipase activity which ultimately may result in measureable enrichment in unsaturated fatty acid composition of beef, and; (3) to evaluate the responses of pure cultures of these bacteria when supplemented with different substrates (corn oil, flaxseed oil, olive oil, and glycerol). The goal of this research was to characterize and measure the growth and lipase activity of each organism when introduced to different triacylglyceride-derived energy substrates.

Results obtained from this research will provide a foundation for further research to development a short-term strategy using glycerol as supplement to finishing diets to inhibit lipolysis activity. Another study would evaluate a means to hyper-expressing the lipase genes from *A. lipolyticus 5S, B. fibrisolvens 49, P. avidum*, or *P. acnes* in *Escherichia coli* for purification of the lipase enzymes. Proof of concept would then be tested by immunizing cattle in an attempt to generate secretory antibodies that will inhibit ruminal lipase activity. With further development these two strategies and implementation of them could have a very profound and positive effect on the beef industry.

CHAPTER II

LITERATURE REVIEW

Digestion and Health Effects of Dietary Lipids

Diets that contain a high content of saturated fats have been associated with a myriad of negative health effects such as increased serum cholesterol levels and risk of coronary heart disease (Wahrburg, 2004). Saturated fatty acids, such as palmitic (C16:0), lauric (C12:0), and mysteric (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 (HDLs) are also affected by saturated fats. High-density lipoproteins are involved in lipid transport and are the predominant dietary mechanism of regulating low density lipoproteins (LDLs) metabolism (Wijendran and Hayes, 2004). The combined effects that saturated fats have on cholesterol are the major driving factors that lead to the ultimate development of health concerns which stresses the importance of reducing saturated fats in daily diets.

Diets that contain a high amount of ω-6 fats increase the plasma ω-6/ ω-3 ratio. Human diets that contain a high ratio of ω-6/ω-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 lowering the intake of ω-6 fats and increasing the intake of ω-3 fats it will aid in decreasing one's risk of developing a chronic disease. The

recommended ratio intake should be between 4:1 and 10:1 (Gebauer et al., 2006; Medicine, 2002).

Absorption of dietary lipids occurs primarily in the small intestine of ruminants. Microorganisms that are found in the rumen 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 fat into saturated fat (Doreau and Chilliard, 1997). Monogastric lack a pre-gastric fermentation organ which carries out lipolysis. Lactating dairy cows, on average, consume around 300-g of linoleic acid daily, of this only about 40 g remain unsaturated and reaches the small intestine intact (Jenkins and Bridges, 2007).

Rumen Microorganisms and Factors that Contribute to Ruminal Lipolysis

The rumen is a self-contained ecosystem where feed consumed by the ruminant is fermented to volatile fatty acids (VFAs) and microbial biomass. The end products serve as the animal's source of energy and protein (Weimer, 1998). There are several different types of micro-organisms found in the rumen that are capable of lipolysis. 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 (Henderson, 1971; Henderson and Hodgkiss, 1973; Polan et al., 1964; Prins et al., 1975).

Anaerovibrio lipolyticus. *Anaerovibrio lipolyticus* is a flagellated, curved rod-shaped anaerobic Gram-negative rumen bacterium that produces an extracellular lipase during exponential growth. Hobson and Mann (1961) first isolated the bacterium from the ovine rumen. The lipase enzyme produce by *A. lipolyticus* is most active at a pH of 7.4 and from 20 to 22ºC. 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 where 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 the 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 (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 triglycerides. Glycerols liberated during hydrolysis from triglycerides are completely metabolized to volatile fatty acids. 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 mL. Prins et al. (1975) found that *A. lipolyticus* was responsible for the major part of the lipolytic activity in ruminant animals.

Interfacial Activation. Lipase activity is increased when at a lipid-water interface. In the absence of this interface, lipases have very little inter-esterifcation 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 mixed and then lyophilized to remove the aqueous and oil phases. From this they were able to compare the 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. Thus, Clarke and Hawke (1970) may not have been able to detect lipolytic activity in the clarified rumen fluid was because there was inadequate interfacial activation to cause sufficient lipase activity. This suggested that the lipase may rather not be cell bound meaning that it is not surrounded by a cell wall. 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.

Butyrivibrio fibrisolvens. High numbers during isolation of *B. fibrisolvens* from ruminal fermentation has indicated that it may also be of major importance in the rumen. *Butyrivibrio fibrisolvens* is an obligate, curved and rod shaped anaerobe 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 polyunsaturated fatty acids (PUFA) to find their way into the meat and milk of ruminant animals (Maia et al., 2010). A study was conducted by Maia et al. (2010) that investigated the mechanisms by which PUFA affects the growth of *B. fibrisolvens* and how they are metabolized by *B. fibrisolvens*. They found linoleic and linolenic acid to lengthen the lag phase of *B. fibrisolvens* with linoleic acid having the greatest effect. However, growth occurred only when PUFA had been converted to vaccenic acid. They also found the major fish oil acids, eicosapentaenoic acid and docosahexaenoic acid (DHA) were not metabolized and prevented growth of *B. fibrisolvens*. From these results they conclude that lipolysis and biohydrogenation occurs 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 were also isolated later on. A 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 grow best in a rumen fluid-glucose medium with 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 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 acnes. *Propionibacterium* 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* strains hydrolyze only neutral lipids but not polar lipids (Cirne et al., 2006; Jarvis et al., 1998; Jarvis et al., 1999; McInerey, 1988). *Propionibacterium* can utilize the glycerol arising from the de-esterification of triglycerides and phospholipids (Jarvis and Moore, 2010).

Propionibacterium avidum is a Gram-positive, rod shaped, facilitative 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 aesculin, 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 facilitative 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 tetracyclines 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 (Chopra and Linton, 1987; Hirashima et al., 1973; Piovant et al., 1978; 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 will be influenced by the type of phospholipid 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* 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 sources 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 were 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, rod-shaped, facultatively anaerobic bacterium. *Propionibacterium acnes* produce 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 exocellular enzyme of different strains of *Propionibacterium*. The results for exocellular 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*.

Linoleic and Linolenic Acid as a Dietary Source

Lipase-expressing bacteria found in the rumen play a major role in the composition and alteration of lipids that enter the rumen so it is important that different avenues be explored in order to protect important lipids in an animal's diet. 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 (C18:2n-6) 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 body's inability to manufacture linoleic acid on its own and it must be supplemented through the diet. It is important in that it is needed for membrane structure of lipoproteins, including HDL.

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. The results showed that when the safflower oil replaced the saturated fatty acid rich coconut oil, the LDL cholesterol production rate decreased 155%-200% resulting in a 75% net decline in low-density lipoprotein cholesterol.

Linoleic and linolenic acid are the most common types of unsaturated fatty acids. They are considered as unsaturated fatty acids by the existence of double bonds in their carbon chain structure. These double bonds, in nature, typically have a *cis* orientation. This configures the hydrogen atoms of the two carbons that are double-bonded on the same side as the fatty acid. Since the hydrogen atoms are located on the same side of 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, resulting when the hydrogen atoms are located on opposite sides of the carbons adjoined by a double bond resulting in a straight configuration. The *trans* orientation is only created when the *cis* double bond configuration is broken and re-created. The micro-organisms discussed earlier are responsible for the breaking of the *cis* configuration that in turn allows for the formation of the *trans* orientation.

Linolenic acid (C18:3n-3) is the second most important essential fatty acid in relation to linoleic acid. Linolenic acid can be elongated and desaturated to form 22-carbon fatty acids in the n-3 family (Wijendran and Hayes, 2004). Seed oils are the richest sources of linolenic acid. Linolenic acid can also be obtained from the thylakoid membranes of green leaves from broad leaf plants therefore providing herbivores with a high source of linolenic acid. Baylin et al. (2003) conducted a case control study in

Costa Rica and found that adipose tissue high in linolenic acid content was associated with lower risk of myocardial infarction.

Conjugated Linoleic Acid. Current nutritional guidelines promote reduced intake of fats, especially saturated fats. *Trans*-10, *cis*-12 conjugated linoleic acid acts as a strong inhibitor of milk fat synthesis and therefore has major potential to be used in the dairy industry in order to reduce levels of milk fat. 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; conjugated linoleic acid can therefore exist in the form of a *trans* fatty acid. Conjugated linoleic acid can be used to supplement dairy cattle diets in order to reduce milk fat; however, conjugated linoleic acid is prone to ruminant degradation. Several strategies have been developed and applied in order to protect conjugated linoleic acid from degradation in the rumen.

Effects of Varying Dietary Supplements on Reducing Lipolysis and Biohydrogenation in the Rumen

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 linolenic acid. Vaccenic acid represents about 60-70% of the *trans* fatty acid in the milk and the 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.

There have been several strategies developed in order to decrease or by-pass 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). 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. There have been a number of studies examining methods in reducing lipolysis and biohydrogenation by supplementing ruminal diets with different lipid sources such as fish, soybean, canola, sunflower, and flaxseed oils (Ashes et al., 1992; Rego et al., 2005; Weill et al., 2002) with each of these studies having varying degrees of success.

Effects of Supplementing Fish Oil in the Rumen. Eicosapentaenoic and docosahexaenoic acid are essential ω-3 fatty acids and are the conversion products of linolenic acid. Mammals are incapable of producing n-3 fatty acids but are capable of

creating long chain unsaturated fatty acids such as eicosapentaenate and docosahexaenate, from short chain n-3 fatty acids such as linolenic acid. Cold-water fish are good sources of eicosapentaenoic and docosahexaenoic acid. These acids are independently associated with an increase in LDL oxidation, 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 eicosapentaenoic and docosahexaenoic acid 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 six-week 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 of eicosapentaenoic and docosahexaenoic acid help decrease the risk of ischemic heart disease. In the 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 eicosapentaenoic and docosahexaenoic acid was not affected; however, biohydrogenation of eicosapentaenoic and docosahexaenoic acid was reduced significantly with increasing fish oil. Linoleic

and linolenic acid appeared to disappear to the same extent. Stearic acid, the end product, 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 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 (Harfoot et al., 1973; Kemp et al., 1984; Kepler et al., 1966). Biohydrogenation of linolenic acid 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 eicosapentaenoic and docosahexaenoic acid 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 eicosapentaenoic and

docosahexaenoic acid 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 eicosapentaenoic and docosahexaenoic acid inhibited linoleic acid isomerase activity of *B. fibrisolvens* (Wasowska et al., 2006).

Effects of Supplementing Vegetable Oil in the Rumen. 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 acids 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.

Effects of Supplementing Sunflower Oil in the Rumen. 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 18-C unsaturated linoleic acid at either level (Sackmann et al., 2003).

Effects of Supplementing Linseed Oil in the Rumen. 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 ω -6/ ω -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 muscle**.**

Effects of Supplementing Glycerol in the Rumen. Research by Krueger et al. (2010) evaluated two levels of glycerol (2% and 20%) and its inhibitory effects on ruminal lipolysis. 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 (Kerley, 2007; Strompl et al., 1999).

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 consistent findings from work already discussed in 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.

Effects of Supplementing Varying Forage Levels in the Rumen. 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. These results suggest that feeding higher levels of forage, it alters ruminal biohydrogenation of linoleic acid, which in turn causes an increased outflow of intermediates.

Technological Advances Used to Protect Lipids in the Rumen

The rapid accumulation of free fatty acids produced by ruminal lipolysis limits the amount of fat that can be introduced into the daily diets of ruminants because of the inhibitory effects free fatty acids 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). As discussed earlier, with supplementing diet, technologies have also been developed to help protect fat sources from ruminal lipolysis and biohydrogenation, such as surrounding unsaturated fatty acids with a protein source that was made resistant to microbial degradation.

Effects of Formaldehyde Cross-Linked Protein Casein in the Rumen. 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 done 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; Delbecchi et al., 2001; Tymchuk et al., 1998). 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.

Effects of Supplementing Calcium Salts in the Rumen. 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 PUFAs. Reviewing both Harvatine and Allen (2006) and de Veth et al. (2005), it can be determined that formaldehyde-protected CLA supplementation is currently the optimal method for use in the dairy industry at lowering milk fat yield.

Effects of 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.

CHAPTER III

DEVELOPMENT OF NON FORAGE BASED INCUBATION SYSTEM FOR CULTURING ANAEROBIC RUMINAL LIPASE-PRODUCING BACTERIA *IN VITRO*

Introduction

Ruminant-derived foods contain high proportions of saturated fats, a result of microbial biohydrogenation within the rumen that rapidly saturates and thus limits the availability of free unsaturated fatty acids for absorption and assimilation. Biohydrogenation cannot occur; however, unless free fatty acids (FFAs) are first hydrolyzed from their triacylglyceride precursors. Consequently, strategies that protect lipids from rumen lipolysis may effectively promote ruminal escape and eventual intestinal absorption of unsaturated fatty acids. This results in the production of value-added ruminant products enriched with healthy unsaturated fats.

As reviewed by Lourenço et al. (2010), the ability of ruminal microbes to hydrolyze triglycerides was reported more than 50 years ago. Since then, numerous studies have characterized the biological and physical factors affecting ruminal lipolysis by mixed or pure populations of ruminal bacteria. For instance, Hawke and Silcock (1970) shown that more than 50% of ruminal lipase activity was contained with the particulate fraction of freshly collected ruminal fluid. Furthermore, microbes attached to digesta were shown to contribute as much as 80% of the biohydrogenation activity in the rumen, presumably because of absorption of the linoleic acid substrate to the particulate

material (Harfoot et al., 1975; Harfoot and Hazlewood, 1997; Harfoot et al., 1973). It has since been recognized that the enzymatic activity of lipases is markedly activated in environments that stabilize the hydrophobic-hydrophilic interface that occurs at the point of contact between oil and water. Thus, 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 lead to considerable variation and experimental error during their use in incubations. Therefore, the objective of the present study was to develop a digesta-free methodology for culturing mixed and pure populations of ruminal lipase-producing bacteria while still providing a solid matrix to support interfacial activation.

Additionally, many previous studies on ruminal lipolysis have been conducted using CO_2 as the anaerobic gas phase. Considering, however, that CO_2 inhibits extracellular lipase production by *Pseudomonas fluorescens* strain B52 (Rowe, 1988) and inhibits the activity of lipase from *Candida rugosa* (Fadíloglu and Erkmen, 2002) as well as biohydrogenation of linoleic acid by mixed populations of rumen bacteria and *B. fibrisolvens* (Polan et al., 1964), this study examined the effects of different gas phases on lipolysis on mix cultures in the incubation system.

Materials and Methods

Mixed Bacterial Populations. Mixed bacterial populations were obtained from a canulated cow maintained on predominantly rye grass pasture. Rumen contents were removed and strained through a nylon paint strainer (Leyendecker et al., 2004) into a pre-warmed insulated container (Thermos®, Rolling Meadows, IL) and transported to the laboratory within 30 min of collection. Upon arrival to the laboratory, CO_2 was bubbled through the ruminal fluid in order to keep it in an anaerobic state.

Cultural Conditions. Mixed bacterial populations obtained from the rumen were cultured in a standard rumen fluid medium containing 150 mL mineral mix one, 150 mL mineral mix two, 100 mL clarified rumen fluid, 1 mL of 0.1% rezazurin, 4 g $NaHCO_3$ and 0.5 g cystine hydrochloride per liter. Mineral mix one contained (per L) 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 $CaCl_2*6H_2O$ and 6 g $CaCl_2$. All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless otherwise noted. The medium was then prepared by boiling to remove dissolved O_2 and then saturated with O_2-free gas while cooled on ice under a continuous flow of 100% CO_2 or, in the case of studies examining the effect of H_2 gas on lipolysis, under a mixture of 50% H_2 in CO_2. The cooled medium was distributed (6 mL/tube) using the anaerobic Hungate technique as described by Bryant (1972) into 18 x 150 mm glass tubes which were immediately closed with rubber stoppers. Tubes were placed in a press to prevent stoppers from being dislodged and sterilized via autoclaving. For tests using glass beads as a solid support matrix, tubes were pre-loaded with approximately 1.7 or 3.5 g per mL media of 4 mm diameter glass

beads (Fisher Scientific, Pittsburgh, PA) prior to addition of medium. After sterilization, 0.1 mL of olive oil was added to tubes cooled to room temperature which were then inoculated with 1 mL freshly collected ruminal fluid and incubated at 39ºC while agitated at 90 rpm in an Innova™ 4000 – Incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). After 48 h of incubation, for maintenance of the culture during the course of the study, transfers were done for a second incubation series to a fresh set of tubes with their given treatment and incubated, for another 48 h. In each experiment tubes for each treatment were given 0.5 mL of concentrated hydrochloric acid (HCL) to stop growth and enzyme activity following inoculation and prior to each incubation series to represent zero time controls. Hydrochloric acid was also distributed to each tube following incubation. Fatty acids were extracted from all tubes and concentrations determined colorimetrically according to methods described by Kwon and Rhee (1986). Following this study 3.5 g of glass beads per mL media and 100% CO_2 was used as the standard for culture techniques in further studies done.

Comparison of Transfer Methods. Following the study with varying levels of glass beads results showed a loss in enzyme activity following the transfer of cultured media to the second incubation series. In accordance with that study 1.0 mL of rumen fluid and olive oil was cultured following the methods described above for mixed cultures with the modification that different transfer methods were assigned to each set of tubes. Two different transfer methods were compared; 1.0 mL of cultured media was transferred and compared to the transfer of 10 glass beads with the use of a disposable inoculating loop in anaerobic conditions using a Bactron Anaerobic Chamber (Sheldon

Labs Manufacturing Inc., Cornelius, OR). Following this study, all transfers were done by transferring 10 glass beads and 1.0 mL cultured media from the first incubation series into the second incubation series.

Comparing Incubation Methods and their Effects on Enzyme Activity. As with the varying glass bead study, 1.0 mL rumen fluid and olive oil were added to tubes and cultured according to the specifications of mixed rumen bacteria discussed above. Treatments were assigned to each set of tubes for incubation with one set being incubated vertically and one horizontally. In further experiments performed in this study, all tubes were incubated at a horizontal angle.

Statistical Analysis. Tests for the effects of the different treatments were done using a general analysis of variance (ANOVA) (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a Tukey's separation of means ($P < 0.05$).

Results and Discussion

Evaluation of Glass Beads and Head Space Gasses. The insolubility of lipid substrates and the lack of interfacial activation are major limitations to the study of ruminal lipolysis in aqueous media. Consequently, many earlier *in vitro* incubations have been conducted using rumen digesta (Garton et al., 1958; Hawke and Silcock, 1970; Krueger et al., 2010; Shorland et al., 1955) or by adding forage substrates to ruminal fluid incubations (Dohme et al., 2003; Van Nevel and Demeyer, 1995). However, the heterogeneous makeup of these contents introduces considerable variability into the conduct of such studies as differences in particle size, chemical composition, stage of

digestion and microbial colonization can markedly affect the amount of surface area available for contact with the lipid substrate. For instance, Krueger et al.(2010) reported rates of ruminal lipolysis of approximately 5060 nmol FFAs liberated/g of undiluted rumen contents per h during a 24 h incubation of 5 g freshly collected rumen digesta with 0.5 g added olive oil. Conversely, based on estimates of amounts of lipid degraded following 24 h incubation of 25 mL freshly collected strained ruminal fluid (lacking particle-associated bacteria), with 0.4 g ground forage and 0.125 g added soy oil, approximately 640 nmol FFA would have been liberated/mL of rumen contents per h (Dohme et al., 2003). In contrast, based on accumulations of free fatty acids reported by Van Nevel and Demeyer (1995) during 6 h incubation of 10 mL freshly collected and filtered rumen fluid diluted with 50 mL buffer containing 0.5 g of a ground concentrate diet and 0.08 g soy oil, the rate of lipolysis was calculated to be approximately 170 nmol FFA/mL per h.

Results from the present study demonstrated that rates of FFA accumulation during incubation of 1 mL freshly collected ruminal fluid in 6 mL of a standard aqueous medium supplemented with 0.1 mL olive oil were markedly lower (less than 3.86 nmol free fatty acid/mL per h) then previous research discussed above. However, during the first incubation series the rates of lipolysis were increased ($P < 0.05$) more than 94% in cultures incubated in tubes containing glass beads compared to rates measured in control cultures incubated without glass beads (Table 3.1). It is possible that the glass beads may have helped to provide an environment conducive to the growth of lipase-producing bacteria as well as for the secretion of the extracellular lipases. In support of this later

hypothesis, Martinez and Nudel (2002) demonstrated that secretion of lipase produced by *Acinetobacter calcoaceticus* was stimulated by glass beads. During the initial incubation series, the rate of lipolysis was more rapid in the tubes containing 3.5 g glass beads per mL medium than in tubes containing only 1.7 g glass beads per mL. The most likely explanation for this difference in rates is that the bed-level in the tubes containing 3.5 g of glass beads per mL media was sufficient to contain all of the medium volume whereas approximately 1.5 to 2 cm of the aqueous medium, of which the added oil floated on top, remained above the bed-level for tubes containing only 1.7 g of glass beads per mL media. Thus, in the tubes containing the higher amount of beads, the oil substrate was spread over the surface and remained in constant contact with glass beads thereby providing an environment favorable for continuous interfacial activation of the extracellular enzymes secreted by lipase-producing bacteria. Work done by Maruyama (2000) compared a interfacially processed lipase to a crude lipase and showed that the interfacially processed lipase had significantly greater inter-esterification activity compared to the crude native lipases.

Table 3.1. Least square mean effect of glass beads and incubation series on ruminal lipase activity *in vitro*.

	Rate of free fatty acid accumulation (nmol/mL/h)[a]		
Incubation series	No Beads	1x Beads	2x Beads
1	3.86^{c}	76.59^{b}	192.34^{a}
2	0.98^{c}	6.70^{c}	7.47^{c}

[a] Values depict least square means calculated from identical replications (n=3). Least square means within rows and columns with unlike superscripts differ at $P < 0.05$. SEM = 13.984.

Rates of FFA accumulation were reduced considerably ($P < 0.05$) after 24 h incubation of a subsequent incubation series conducted likewise, but inoculated with 1 mL of fluid from respective cultures of the first incubation series. Moreover, the rates did not differ ($P > 0.05$) between cultures incubated with or without the inclusion of beads in the second incubation series. The loss of lipase activity in the second incubation series suggests that lipase-producing bacteria in the cultures from the first incubation series were not planktonic or free floating, but rather were attached or firmly associated with the bead environment.

The highest rate of FFA accumulation was achieved in the full bead treatment when all liquid contents of the test tube were completely submerged just under the bead level suggesting that the full bead treatment provided the most contact between the bacteria and their substrate. The glass beads helped in dispersing the oil throughout the medium and the limitation of using a lower level of bead treatment is that the oil can still separate from the medium and coalesce together. Since the full bead treatment provided the maximal interfacial activation in this trial, this treatment would be applied as a standard in further assays conducted in this study.

The study done by Polan et al. (1964) showed that more biohydrogenation was achieved when *B. fibrisolvens* was exposed to an atmosphere of hydrogen while an atmosphere of carbon dioxide decreased activity. Treatments of CO_2 and a H_2:CO_2 (50:50) mix were incorporated with the bead variation trial to determine if these treatments would cause similar effects on ruminal mixed culture bacterial lipase activity. Results revealed no difference ($P > 0.05$) in lipase activity due to the composition of

head space gasses used between treatments with mean rates being 78.00 and 103.87 (SEM = 12.419) during the first incubation series and 4.37 and 5.73 (SEM = 16.032 for tubes incubated under CO_2 or H_2:CO_2, respectively. Based on the results of this study in combination with the work done by Polan et al. (1964), it can be suggested that different headspace gasses only have an effect during the biohydrogenation step of lipid saturation.

Tube Orientations During Incubation. Different tube orientations were compared during incubation and tubes were agitated in attempts to increase surface area in which the medium and energy substrate could interact with the glass beads. Tube sets were incubated vertically and horizontally; results (Figure 3.1) showed that there was an increase in rates of FFA accumulation (nmol/mL per h) in the treatment that was incubated horizontally. Although the effect of horizontal tube orientation on enzyme rate was non-significant ($P > 0.05$), all reaction tubes were incubated horizontally in subsequent studies.

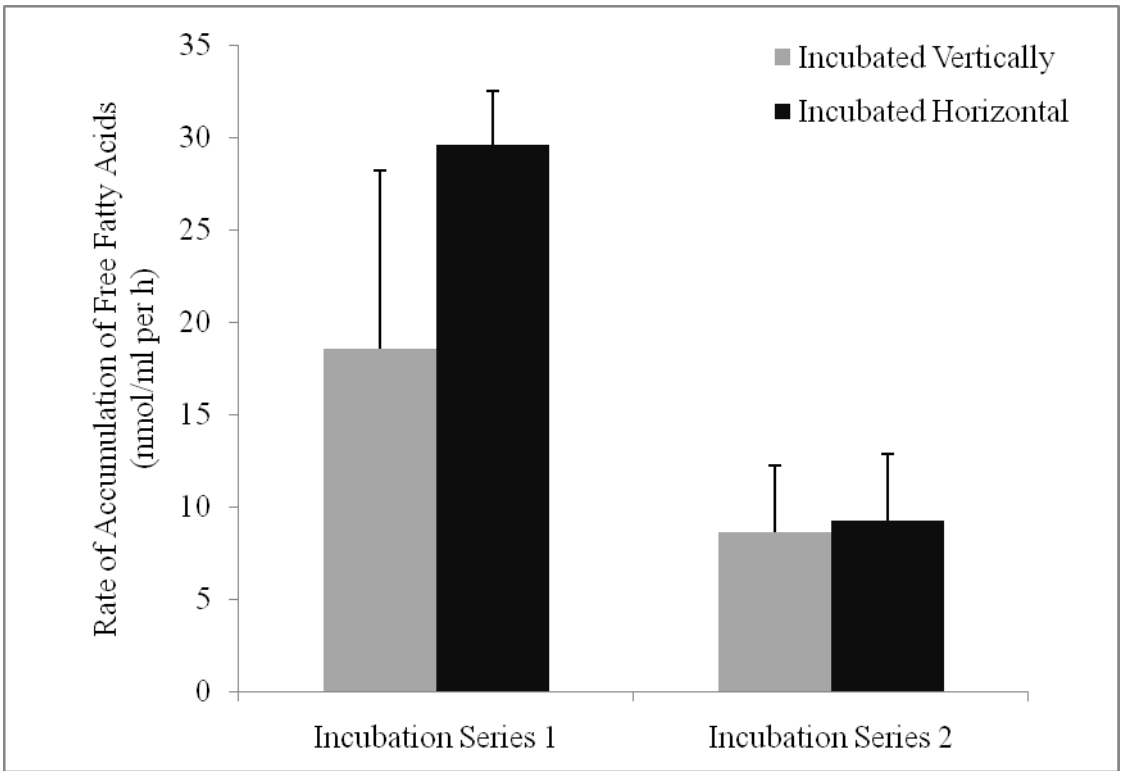

Figure 3.1. Comparison of rates of free fatty acid accumulation by mixed population of ruminal microbes incubated 48 h at 39°C. Test tube sets were incubating vertically or horizontally; bars indicate means of three replications with error bars indicating one standard deviation from the mean.

Transfer Techniques. Results from the glass beads study showed that there was a significant loss of lipase activity when the media were transferred from the first incubation series to the second incubation series. The loss in activity after the transfer is detrimental to obtaining accurate results from assays performed later in the study because transfers are needed to carry out the experiments. It was considered that the presence of the glass beads served as a support matrix simulating digesta found in the rumen and therefore the bacteria could potentially be adhering to the glass beads and not be present in a planktonic state. The transfer of planktonic bacteria between incubation

series of mixed rumen cultures was compared to the transfer of just the glass beads. The results (Figure 3.2) revealed a tendency ($P = 0.061$) for the bead transfer to support higher subsequent rates versus fluid transfer as rates were increased >79% upon transfer of beads, but were decreased 34% upon transfer of fluid. These findings indicated that accumulation of free fatty acids (nmol/mL per h) was higher for the tubes containing previously-incubated beads versus those inoculated only with previously-inoculated fluid medium.

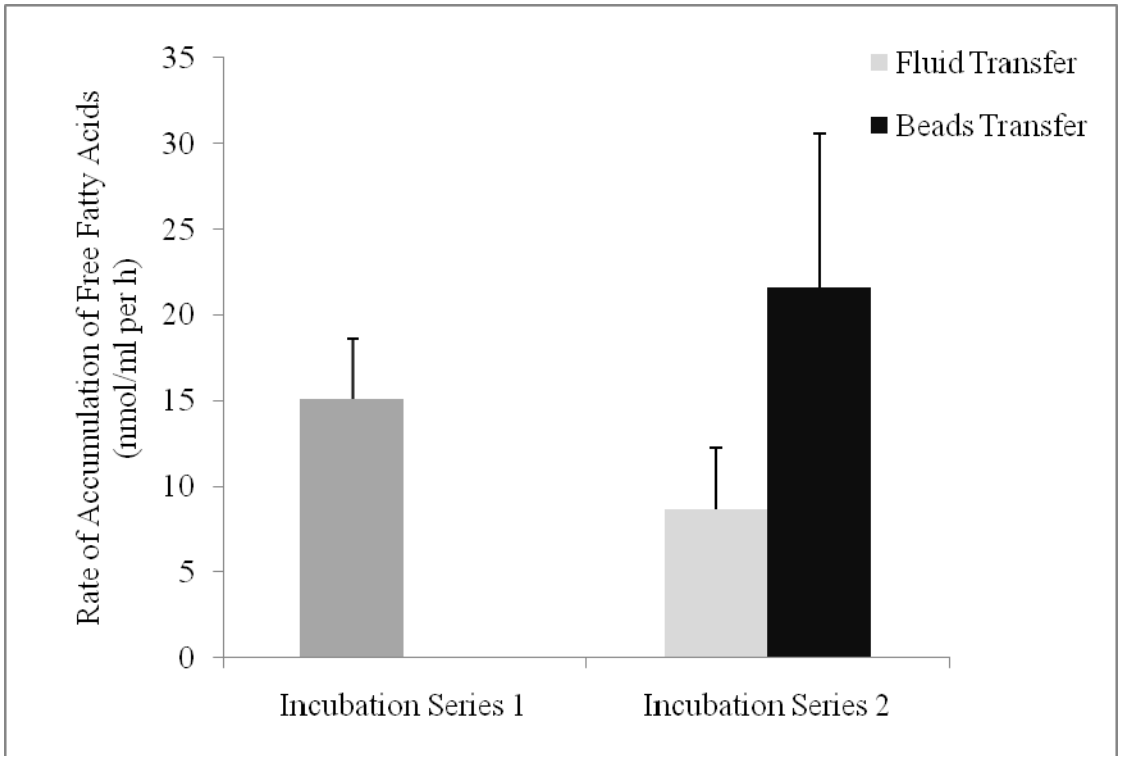

Figure 3.2. Comparison of free fatty acid accumulation by mixed population of ruminal microbes incubated 48 h at 39°C. Beads or fluid fraction from test tube sets were transferred between incubation series; bars indicate means of three replications with error bars indicating one standard deviation from the mean.

Implications

Results showed that the introduction of glass beads into an *in vitro* incubation system markedly increased lipolysis, most likely a result of establishing a better interface between the aqueous medium and the lipidic, energy substrate. The introduction of a second incubation series demonstrated that the ruminal bacteria were adhering to the glass beads, suggesting that along with increasing interfacial activation the glass beads served as a solid support matrix simulating rumen digesta. The use of glass beads in place of fiber digesta, in incubation systems, allows for a more controlled system during culturing whereas fiber digesta is a more heterogeneous solid support matrix that differs in degree of degradation, chemical composition and microbial colonization.

Previous work has implicated CO_2 as having an inhibitory effect on lipase-producing bacteria. Therefore, the use of different headspace gasses was compared and results showed that in mixed rumen populations the use of 100% CO_2 did not demonstrate an inhibitory effect when compared to a combination of H_2:CO_2 (50:50). The application of CO_2 into the incubation system allowed for the production of comparable results to other ruminal studies that have traditionally used CO_2 to establish anaerobic conditions.

CHAPTER IV

GLYCEROL INHIBITION OF RUMINAL LIPOLYSIS *IN VITRO*

Introduction

Glycerol is a major by-product in the production of biodiesel. Recently glycerol has been investigated for use as a dietary supplement for ruminants as a means to inhibit lipolysis, a pre-requisite of biohydrogenation. Work done *in vitro* by Krueger et al. (2010) showed that supplemental glycerol inhibited ruminal lipolysis by 60-80% when introduced at an inclusion rate of 2 and 20% of dry matter. However, results from this study also showed glycerol content exceeding 20% of dry matter resulted in neutral detergent fiber digestion being negatively affected. Gilbery et al. (2010) showed that glycerol concentrations exceeding 6% negatively affected acid detergent fiber and neutral detergent fiber digestion. Our goal was to investigate whether FFA accumulation could still be reduced at the 6% inclusion rate and also to evaluate and compare free fatty acid accumulation between the 6% and 20% concentration levels.

Ruminal lipolysis has long been attributed mainly to *A. lipolyticus 5S* and *Butyrivibrio fibrisolvens 49*. Conversely, *P. avidum* and *P. acnes* are also known to express lipase activity but little is known regarding the contribution of these prominent anaerobes to rumen lipolysis. Two potential mechanisms of glycerol's inhibition on microbial lipase activity were examined in this study in an attempt to characterize these four bacteria organisms and their role in the rumen. A electron microscope study done by Henderson (1973) showed that the lipase produced by *A. lipolyticus* is extracellular.

Similar studies were conducted on *B. fibrisolvens*, *P. avidum*, *and P. acnes* and results indicated concluded that all of them produce an extracellular lipase (Cotta and Hespell, 1986; Greenman et al., 1983; Miskin et al., 1997). Prins et al. (1975) showed that the lipase was constitutively expressed when cultured *in vitro*. Some ruminal lipase-producing bacteria, such as *A. lipolyticus 5S,* target the glycerol backbone, freeing the esterified fatty acids leaving the acids susceptible to eventual biohydrogenation. Other bacteria, such as *B. fibrisolvens 49* may be fatty acid auxotrophs and thus express lipase so as to acquire free fatty acids required for membrane construction. Because glycerol is one of the products of lipolysis, it was thus hypothesized that supplementing glycerol to culture media would effectively inhibit lipolysis through the means of displaced equilibrium in the breakdown reaction of a triacylglyceride.

A study was also conducted to examine the induction of the lipase in each of these bacteria. The bacterium's DNA carries the template for the lipase gene and from there it is transcribed into mRNA. The coded information for the lipase gene is carried in mRNA where it can later be translated into a functional protein. It was hypothesized that glycerol may causes the repressor molecule to bind to the lac operon preventing RNA polymerase from binding and transcription taking place. By gaining better understanding of glycerol's role in the inhibition of lipolysis, better strategies can be developed for inhibiting lipolysis in the rumen.

Materials and Methods

Mixed and Pure Bacterial Populations. Ruminal fluid containing a mixed bacterial population was obtained from a canulated cow maintained on predominantly rye grass pasture. Rumen content was removed and the fluid strained through a nylon paint strainer (Leyendecker et al., 2004) where it was collected into a pre-warmed insulated container (Thermos®, Rolling Meadows, IL) for transport to the laboratory. Immediately following transportation, CO_2 was bubbled through the rumen fluid in order to keep it in an anaerobic state. Pure culture strains of *A. lipolyticus 5S* and *B. fibrisolvens 49* were obtained from Dr. Jay Yankee, Agriculture-Agri Food Canada, (Lethbridge, Alberta). Strains of *P. avidum* and *P. acnes* were previously isolated from the rumen of a pastured cow (Krueger et al., 2008) and had been found to exhibit among the highest rates of lipolytic activity in earlier screening studies. For long-term preservation of the pure cultures, these bacteria were stored in 20% anaerobic glycerol at - 80ºC.

Glycerol Inhibition on Mixed Ruminal Cultures. Using the Hungate technique described by Bryant (1972), 5 mL of standard anaerobic medium which contained (per liter) 292 mg of K_2HPO_4, 292 mg of $KHPO_4$, 480 mg $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_20$, 4,000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 10 g of trypticase (BBL Microbiology Systems, Cockeysville, MD), 2.5 g of yeast extract, branched-chain fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and trace minerals (Cotta and Russell, 1982). All chemicals were purchased from Sigma-Aldrich

(Milwaukee, WI) unless otherwise noted. Medium was boiled to displace dissolved oxygen, then cooled on ice while being saturated with a continuous flow O_2-free CO_2 and then distributed to 18 x 150 mm glass tubes using the anaerobic Hungate technique as described by (Bryant, 1972). Tubes were assigned glycerol treatments of the following concentrations: 0%, 6%, and 20% (vol/vol). Along with glycerol, tubes also contained 0.1 mL olive oil and 2 mL of mixed culture rumen fluid was used to inoculate each tube. Tubes collected from each treatment immediately upon inoculation with ruminal fluid received 0.5 mL of concentrated HCL, to represent the zero time controls, in order to stop growth and lipase activity. The remaining tubes were incubated and agitated for 48 h in an Innova™ 4000 – Incubator shaker (New Brunswick Scientific Co. Inc., Edison, NJ) at 39ºC. The incubation tubes were then also given 0.5 mL of concentrated HCL and total FFAs were extracted and concentrations determined colorimetrically according to methods described by Kwon and Rhee (1986).

Cultural Conditions for Pure Culture Bacteria. Upon removal from storage, each bacterium was revived during two consecutive 24 to 48 h cultures in 10 mL standard anaerobic medium supplemented with 2% pre-sterilized olive oil. Each bacteria was then grown for a third consecutive culture in standard anaerobic medium supplemented with pre-sterilized olive oil, glucose or glycerol as indicated. Tubes were incubated and agitated horizontally for 24 to 96 h as indicated in an Innova™ 4000 – Incubator shaker.

Effects of Lipid or Non-Lipid Energy Sources on Growth of Pure Cultures of Rumen Lipolytic Bacteria. Effects of lipid or non-lipid energy sources on growth of

the ruminal lipase-producing bacteria *A. lipolyticus 5S, B. fibrisolvens 49, P. avidum*, and *P. acnes* were determined by inoculating 0.2 mL of a late log phase growth culture into 4 mL of the same anaerobic standard medium containing treatments of 5% olive oil and 0.02% glucose. *Anaerovibrio lipolyticus 5S* is unable to utilize glucose as an energy source so it was grown in 5% glycerol in place of glucose. The bacteria were incubated at 39ºC in an L-C Incubator and removed every 6 h over the course of 4 d to be measured for growth. Bacterial growth was determined by measuring absorbance at 600 nm (A600) on a spectrophotometer (Spectronic 20D+, Spectronic Instruments, Inc., Rochester, NY). Mean specific growth rates, μ, were calculated according to the equation ($\mu = \Delta \log_{10} A600/\Delta t$, where t = time) (Koch, 1981).

Assay Conditions. Short term anaerobic assays were conducted to minimize effects of cell propagation. Potential induction of enzymatic activity was determined in 18 x 150 mm glass tubes preloaded with 21 g solid 4 mm glass beads, and additions of 0.6 mL olive oil and anaerobic buffer were made to achieve a final reaction volume of 6 mL. The anaerobic buffer contained only minerals and cysteine-HCl as in the standard buffer but otherwise was prepared similarly.

Test for Glycerol's Mechanism of Lipolysis Inhibition. In the first enzymatic assay conducted to test if displaced equilibrium would affect lipase activity. Cells were obtained from cultures grown to late logarithmic phase with olive oil as the only added energy substrate. The entire contents of cultures containing 2 or 4 mL volume were transferred anaerobically to the assay tubes prepared as above so as to ensure transfer of soluble and cell-associated lipase enzymes, thereby representing 1X and 2X crude

enzyme suspensions in the final reaction volume which was achieved upon addition of anaerobic buffer and 0, 2 or 10% added glycerol. A 0.2 mL sample was taken from each tube for determination of protein using the Modified Lowry Protein Assay Kit (Pierce, Rockford, IL) and then tubes were incubated at 39^{o}C and agitated horizontally in an Innova™ 4000 – Incubator shaker. Reactions were stopped at 0 and 8 h by addition of 0.5 mL concentrated HCl and FFA extractions and measurements were performed as described earlier.

In a second enzymatic assay conducted to test if energy substrate during growth may influence, via induction, lipase activity during subsequent assay with olive oil, each respective bacterium was grown in 4 mL of standard anaerobic medium supplemented with either 5% olive oil or with a non-lipid energy source. For *B. fibrisolvens 49*, *P. avidum* and *P. acnes*, the non-lipid energy source was provided by 0.2% glucose but for *A. lipolyticus 5S* 5% glycerol was provided as this bacterium cannot ferment glucose. Cells obtained from cultures grown to late log phase were adjusted to achieve equivalent A600 via dilution with anaerobic buffer and then the contents of each tube were transferred anaerobically into assay tubes prepared as described above. Assay tubes were incubated as described above and reactions were stopped at 0 and 6 h by addition of 0.5 mL concentrated HCl for subsequent FFA extractions and measurement.

Statistical Analysis. Tests for the effects of the different treatments were done using a general analysis of variance (ANOVA) (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a Tukey's separation of means ($P < 0.05$).

Results and Discussion

Glycerol Inhibition on Mixed Ruminal Cultures. Consistent with the earlier observations of Krueger et al. (2010), results from the present study showed that rates of FFA accumulation (nmol/mL per h) were reduced more than 80% by glycerol treatment when compared to controls (8.606 nmol/mL per h). Furthermore, results indicated that the rate of FFA accumulation observed with the 6% glycerol treatment (1.673 nmol/mL per h) did not differ ($P > 0.05$) from that observed with the 20% glycerol treatment (0.4866 nmol/mL per h; SEM = 1.258). Considering findings of Gilbery et al. (2010), the results obtained here suggest that supplementing ruminant diets with 6% glycerol would be sufficient to effectively reduce rumen lipolysis without adversely affecting digestion.

Effects of Lipid or Non-Lipid Energy Sources on Growth of Pure Cultures of Rumen Lipolytic Bacteria. In order to examine the effects of lipid or non-lipid energy sources on growth of *A. lipolyticus 5S, B. fibrisolvens 49, P. avidum*, and *P. acnes,* growth rates and maximum absorbencies were measured during their culture in media supplemented with olive oil or glucose, or in the case of *A. lipolyticus 5S*, with glycerol. Each bacterium was analyzed separately with measurements being taken for rate and maximum growth.

The results (Tables 4.1 and 4.2) indicate that olive oil supplementation produced significantly higher maximum growth, as evidenced by a higher absorbance, for *A. lipolyticus 5S* but the growth rate was significantly faster when grown in glycerol ($P < 0.05$) (Table 4.1). *Butyrivibrio fibrisolvens 49* had a significantly higher maximum

Table 4.1. Least square means of the mean specific growth rate during growth of ruminal lipase-producing bacteria with lipid or non-lipid energy source.

	Mean specific growth rate (μ)			
Growth substrate	*A. lipolyticus 5S*	*B. fibrisolvens 49*	*P. avidum*	*P. acnes*
5% Glycerol	0.0664	---	---	---
0.02% Glucose	---	0.0505	0.0566	0.0406
5% Olive oil	0.0130	0.0162	0.0293	0.0234
Main effect	----------	----------	----------	----------
Growth substrate	$P = 0.0001$	$P = 0.0447$	$P = 0.0074$	$P = 0.1260$
CVC[a]	0.0097	0.0330	0.0151	0.0229
SEM	0.0025	0.0084	0.0038	0.0063

[a]CVC: critical value for comparison (equivalent to a Fishers Protected LSD and represents the difference between means needed to achieve significance at $P < 0.05$).

Table 4.2. Least square means of the maximum observed absorbance during growth of ruminal lipase-producing bacteria with lipid or non-lipid energy source.

Growth substrate	Maximum observed absorbance (A600 nm)			
	A. lipolyticus 5S	*B. fibrisolvens 49*	*P. avidum*	*P. acnes*
5% Glycerol	1.2267	---	---	---
0.02% Glucose	---	1.9990	1.9990	1.9990
5% Olive oil	1.9990	0.4750	1.9990	1.4430
Main effect	----------	----------	----------	----------
Growth substrate	$P < 0.0001$	$P = 0.0003$	$P = 1.000$	$P = 0.1224$
CVC[a]	0.0334	0.3655	0.0001	0.7908
SEM	0.0085	0.0930	0.0001	0.0248

[a]CVC: critical value for comparison (equivalent to a Fishers Protected LSD and represents the difference between means needed to achieve significance at $P < 0.05$).

growth for both glucose treatments than for the olive oil treatments (Table 4.2). This is not surprising, as while *B. fibrisolvens 49* can obtain energy for growth by fermenting a wide variety of sugar and amino acid substrates, it does not ferment glycerol and likely would gain little energetic benefit from high lipase activity. Some strains of *B. fibrisolvens* are auxotrophic for fatty acids and thus lipase activity may provide a mechanism to acquire fatty acids needed for their cell membrane synthesis (Hazlewood et al., 1980). The results also indicated that there was not a significant difference for the growth rate of *B. fibrisolvens 49* amongst both treatments, with amino acid sources likely serving as fermentable substrate for growth in the olive-oil supplemented medium. The glucose treatment for *P. avidum* showed a significantly higher growth rate for glucose than olive oil; however, there was not a significant difference in the results for maximum growth between the two treatments for *P. avidum*. The results for *P. acnes* did not indicate significant differences between the three treatments for either maximum growth or growth rate. The results for *P. acnes* did not indicate significant differences between the two treatments for either maximum growth or growth rate.

Test for Potential Displaced Equilibrium Effect. Glycerol was tested to determine if it causes an equilibrium displacement in the triacylglyceride reaction resulting in an inhibition in lipase activity. The enzymatic ability of entire contents of cultures (2 or 4 mL) of *A. lipolyticus 5S, B. fibrisolvens 49, P. avidum* and *P. acnes* to hydrolyze olive oil (10%) was assayed in the presence of 0, 2 or 10% added glycerol. Tubes were pre-loaded with glass beads which served as a reaction matrix. The entire contents of the cultures were used in the short-term assay so as to include lipase that

could be both soluble and or cell-associated. An effect of glycerol treatment was not observed on any of the bacterial suspensions thus indicating that glycerol had no direct inhibitory effect on lipase activity by these pure cultures (Table 4.3). In the case of *B. fibrisolvens 49, P. avidum* and *P. acnes*, but not *A. lipolyticus 5S*, a main effect of enzyme concentration was observed, with more activity observed in assays containing 2X bacterial suspension than 1X bacterial suspension (Table 4.3). Provided olive oil was present in excess during the assay it would be expected that more enzyme in the reaction mixture would yield higher activity. Only in the case of *B. fibrisolvens 49* was a glycerol by enzyme level interaction observed (Table 4.3).

Glycerol's Down Regulation on Gene Expression. For this study each of the four bacteria were grown in either glucose or olive oil with the exception of *A. lipolyticus 5S* which was grown in glycerol in place of glucose due to its inability to effectively utilize glucose as an energy substrate. With the lack of oil as a substrate in the glucose/glycerol grown treatments it was hypothesized that the bacteria would not produce the lipase enzyme effectively inhibiting lipolysis. The results (Table 4.4) suggest that lipase activity by *A. lipolyticus 5S* and *P. avidum* was not regulated by glucose or glycerol, as specific activities (nmol FFA/mg protein per h) by cells grown with these substrates were not decreased ($P > 0.05$) compared to activities by cells grown with olive oil. There was a significant effect of energy substrate on specific activity obtained with *P. avidum* but the activity was increased in cells grown with glucose compared to cells grown with olive oil, which certainly indicates that glucose did not down regulate expression of lipase activity by this bacterium (Table 4.4).

Table 4.3. Least square means of the specific lipase activity of olive oil grown cells assayed in the presence of olive oil (10%) with 0, 2 or 10% added glycerol as potential inhibitor.

Amount of added glycerol (vol/vol)	Specific activity (nmol free fatty acid/mg protein per h)							
	A. lipolyticus 5S		*B. fibrisolvens 49*		*P. avidum*		*P. acnes*	
	1X protein	2X protein	1X protein	2X protein	1X protein	2X protein	1X protein	2X protein
0	0.0031	0.0024	0.0230	0.0302	0.0219	0.0878	0.0170	0.0535
2%	0.0052	0.0060	0.0424	0.0378	0.0459	0.0758	0.0302	0.0663
10%	0.00090	0.0051	0.0063	0.0531	0.0115	0.1356	0.0077	0.0579
Main Effects	----------		----------		----------		----------	
Glycerol	$P = 0.4898$		$P = 0.3395$		$P = 0.7449$		$P = 0.3976$	
Protein level	$P = 0.4590$		$P = 0.0492$		$P = 0.0033$		$P = 0.0014$	
Interaction	$P = 0.5781$		$P = 0.0399$		$P = 0.1946$		$P = 0.8124$	
CVC[a]	0.0130		0.0437		0.1166		0.0614	
SEM	0.0024		0.0092		0.0245		0.0139	

[a]CVC: critical value for comparison (equivalent to a Fishers Protected LSD and represents the difference between means needed to achieve significance at $P < 0.05$).

Table 4.4. Least square means of the specific lipase activity of lipid or non-lipid energy source grown cells assayed in the presence of olive oil (10%).

Growth substrate	Specific activity (nmol free fatty acid/mg protein per h)			
	A. lipolyticus 5S	*B. fibrisolvens 49*	*P. avidum*	*P. acnes*
Glycerol	0.2080	---	---	---
Glucose	---	0.0001	0.4511	0.0273
Olive oil	0.3607	0.0544	0.3019	0.0666
Main effect	----------	----------	----------	----------
Growth substrate	$P = 0.5161$	$P = 0.0518$	$P = 0.3820$	$P = 0.0135$
CVC[a]	0.5967	0.0551	0.1363	0.0259
SEM	0.1518	0.0140	0.0347	0.0065

[a]CVC: critical value for comparison (equivalent to a Fishers Protected LSD and represents the difference between means needed to achieve significance at $P < 0.05$).

Regardless of growth substrate, *P. acnes* and *B. fibrisolvens 49* cells expressed lower lipase activity ($P < 0.05$) than *A. lipolyticus 5S* and *P. avidum.* In the case of glucose-grown *P. acnes* cells, lipase activity was lower ($P < 0.05$) than olive oil-grown cells thus suggesting that glucose down regulated lipase gene expression by bacterium (Table 4.2). Similarly, there was a tendency ($P < 0.10$) for *B. fibrisolvens 49* cells grown with glucose to express lower specific lipase activity than olive oil grown cells. Results indicate that higher and constitutively expressed lipase activity of *A. lipolyticus 5S* and *P. avidum* probably contribute more to lipolysis of triacylglycerol in ruminants than *P. acnes* and *B. fibrisolvens 49.* There is a conundrum in that activities of *A. lipolyticus 5S* and *P. avidum* that were unaffected by glycerol which suggests that there may be an even more important as yet unidentified glycerol-susceptible rumen bacterium contributing to rumen lipolysis.

Implications

Based on the results in this set of experiments there does not appear to be a significant justification for supplementing glycerol above the 6% concentration in the ruminal diet which, based from other glycerol research, should prevent any negative effects that glycerol may have on neutral detergent fiber digestion. However, further research needs to be done to determine the effects of feeding glycerol for different lengths of time in the finishing diet and what the result may be by withdrawing glycerol from the finishing diet after introducing it for an applicable length of time. By conducting further research the best application could be suggested for establishing a

time frame for feeding increasing amounts of glycerol in order to achieve the maximum reduction in the accumulation of free fatty acids.

Further characterization and understanding of pure culture bacteria supplemented with glycerol has provided insight in furthering the manipulation of glycerol's effect on these bacteria and the development of strategies for inhibiting lipolysis in the rumen. Our investigation of lipase gene expression did not contain measurements of mRNA; therefore, the results do not provide certainty that lipase gene expression is down regulated in *B. fibrisolvens 49* and *P. acnes*. However, it is likely that lipolysis by *B. fibrisolvens 49* and *P. acnes* were not inhibited by the mechanism of equilibrium displacement and that it is most likely by the interruption of the transcription process of mRNA. *Propionibacterium avidum* also consistently showed the highest lipolysis activity in these studies. Based on this knowledge our attention is being directed towards trying to inhibit the lipase activity of *A. lipolyticus 5S* and *P. avidum.*

CHAPTER V

VARYING ENERGY SUBSTRATES AND THEIR EFFECTS ON PURE CULUTRES OF RUMINAL LIPASE-PRODUCING BACTERIA

Introduction

Diets that contain a high amount of saturated fats have been associated with negative health effects such as increased serum cholesterol levels and risk of coronary heart disease (Wahrburg, 2004). Food products derived from ruminant animals contain high contents of saturated fats, a consequence of microbial biohydrogenation. Biohydrogenation is a process that occurs in the rumen that rapidly saturates FFAs, thus limiting the availability of free unsaturated fatty acids for absorption and assimilation. In order for biohydrogenation to occur FFA must first be hydrolyzed from their triacylglyceride precursors. Consequently, strategies that protect lipids from rumen lipolysis may effectively promote ruminal escape and intestinal absorption of unsaturated fatty acids.

Ruminal lipolysis has long been attributed mainly to *A. lipolyticus* and *B. fibrisolvens*. Conversely, *Propionibacterium* species *avidum* and *acnes* are also known to express lipase activity but little is known regarding the contribution of these prominent anaerobes to rumen lipolysis. Moreover, it is known that the introduction of different energy substrates can have mixed effects on the growth and metabolic activity of ruminal bacteria and extensive research has been done to try and develop a dietary regimen that can lower the impacts of lipolysis and biohydrogenation in the rumen.

Flaxseed oil, also known as linseed oil, is rich in α-linolenic acid which is an ω-3 fatty acid found predominantly in plants. Weill et al. (2002) studied the protective effect that linseed has on fatty acids when introduced to a livestock diet and results showed that use of linseed oil decreased the fat percentage in milk; however, it did not have an effect on daily fat production. Morris (2008a) demonstrated the effects of introducing linseed into beef cattle diets and showed that linseed oil increases α-linolenic acid and CLA content in beef adipose tissue which consequently decreased the ω-6/ω-3 ratio. In order to further characterize and understand the lipase activity of these bacteria, each was grown with four different energy substrates: olive oil, corn oil, flaxseed oil, and glycerol. Results from this study will help elucidate the potential contribution of each of these bacteria to the lipolysis of differing lipid sources potentially entering the rumen. Ultimately, the development of a better dietary ration will help capitalize on other strategies developed for the reduction of lipolysis.

Materials and Methods

Pure Bacterial Populations. Pure culture stains of *A. lipolyticus 5S* and *B. fibrisolvens 49* was obtained from Dr. Jay Yankee, Agriculture-Agri Food Canada (Lethbridge, Alberta). 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 the pure cultures, these bacteria were stored in 20% anaerobic glycerol at -80ºC.

Cultural Conditions for Pure Culture Bacteria. Bacteria were grown in standard anaerobic medium under 100% CO_2 containing (per L) 292 mg of K_2HPO_4, 292

mg of $KHPO_4$, 480 mg $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_20$, 4,000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 10 g of trypticase (BBL Microbiology Systems, Cokeysville, Md.), 2.5 g of yeast extract, branched-chain fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and trace minerals (Cotta and Russell, 1982). All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless indicated otherwise. Glucose or glycerol, when utilized, was added before autoclaving to achieve 0.2 mg or 3.6 mg/mL final concentration, respectively. The medium was then further prepared by boiling to remove dissolved O_2 and then saturated with O_2-free gas while cooled on ice under a continuous flow of 100% CO_2. The cooled medium was distributed (6 mL/tube) using the anaerobic Hungate technique as described by Bryant (1972) to 18 x 150 mm glass tubes which were immediately closed with rubber stoppers. Tubes were placed in a press to prevent stoppers from being dislodged and sterilized via autoclaving. Tubes were cooled to room temperature before inoculation. Following inoculation with the given bacteria tubes were incubated and agitated horizontally for a given amount of time in an Innova™ 4000 – Incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ) at 39ºC at 90 rpm. In each experiment tubes for each treatment were given 0.5 mL of concentrated HCL to stop growth and enzyme activity following inoculation and prior to each incubation series to represent zero time controls. Hydrochloric acid was also distributed to each tube following incubation. Fatty acid extractions were performed on all tubes according to methods described by Kwon and Rhee (1986).

Growth Curves. Growth curves were performed on the pure culture strains in preparation for the study comparing different energy substrates. This was done to determine mid logarithmic and stationary phase of growth of each bacteria in the presence of each energy substrate. Each bacterial strain, following removal from -80ºC storage, was cycled twice in 10 mL of the standard anaerobic media containing 0.2 mL olive oil before beginning the study. To determine the growth curve, 0.2 mL of each bacterium was transferred anaerobically to 6 mL of the same anaerobic standard medium containing treatments of the four different energy substrates (3% vol/vol) and absorbance at 600 nm (A600) readings were taken every 6 h on a Spectronic 20D+spectrophotometer (Spectronic Instruments Inc., Rochester, NY). Specific growth rate, μ, was calculated according to the equation ($\mu = \Delta \log_{10} A600/\Delta t$, where t = time) (Koch, 1981). Growth curve procedures were repeated with the modification that glucose was absent from the media.

Energy Substrate Comparison. Each pure culture bacterium was grown in 6 mL of standard anaerobic medium containing 3% of each energy medium treatment. The bacteria were cultured according to previously elaborated methods for pure culture bacteria. Growth for each bacterium in each energy substrate was stopped at zero time, early log, and stationary growth phases with each treatment and time phase being done in triplicate. This experiment was repeated with the modification that glucose was left out of the standard anaerobic medium.

Statistical Analysis. Tests for the effects of the different treatments were done using a general analysis of variance (ANOVA) (Statistix v.9.0, Analytical Software, Tallahassee, FL) with a Tukey's separation of means ($P < 0.05$).

Results and Discussion

Growth Curves. *Anaerovibrio lipolyticus 5S* and some *Butyrivibrio* species have long been considered to play a major role in the ruminal hydrolysis of dietary lipids, with *A. lipolyticus 5S* contributing mainly to the hydrolysis of triglycerides and *B. fibrisolvens 49* contributing mainly but not exclusively to the hydrolysis of galactolipids and phospholipids (Lourenço et al., 2010). More recently, isolations of lipolytic clostridia, propionibacteria, staphylococci and selenomonads from the rumen have been reported (Cirne et al., 2006; Dighe et al., 1998; Jarvis and Moore, 2010; Krueger et al., 2010). There have been very few recent studies comparing the effects of different energy substrates on the growth characteristics and lipolytic activities of these prominent lipolytic bacteria.

Growth curves were performed measuring growth rate (Table 5.1) and maximum absorbance (Table 5.2) of *A. lipolyticus 5S*, *B. fibrisolvens 49*, *P. avidum*, and *P. acnes* in the presence or absence of glucose in four varying energy substrates. Each oil substrate and glucose treatment was analyzed separately to determine if there was an interaction between the bacteria. *Propionibacterium acnes* and *avidum* exhibited a much broader substrate range than *A. lipolyticus 5S*. Both species of *Propionibacterium* differ from *B. fibrisolvens 49* in their ability to ferment glycerol (Holdeman et al., 1977;

Stackebrandt and Schaal, 2006) and thus it is reasonable to hypothesize that they may hydrolyze triacylglycerides to access glycerol. However, the possibility that some of the liberated fatty acids may be assimilated into lipid membrane of these bacteria cannot be excluded. The results indicate that *P. acnes* and *P. avidum* generally grew the most rapid in every oil and glucose treatment when compared to the other bacterium (Table 5.1). However, the exception of this was *P. avidum* which did not grow the most rapid ($P > 0.05$) when grown in flaxseed oil compared to the other bacteria. In contrast to this *P. acnes* in that same treatment had most rapid rate of growth ($\mu = 0.1100$). *Propionibacterium avidum* supported the highest cell density consistently across all treatments when compared to the other bacteria. *Propionibacterium acnes,* however, consistently supported the lowest ($P < 0.05$) cell density among all treatments.

Butyrivibrio fibrisolvens 49 also possesses a broad substrate profile, being able to catabolize and ferment a variety of polysaccharide and protein substrates. Nevertheless, many strains of *B. fibrisolvens,* including strain 49, do not ferment glycerol (Bryant and Small, 1956). *Butyrivibrio fibrisolvens 49* thus likely expressed extracellular lipase to acquire nutritional sources of medium and long chain fatty acids for their cell membranes (Hazlewood et al., 1980). The growth curve results showed that *B. fibrisolvens* grew the slowest consistently when compared to the other bacterium. *Anaerovibrio lipolyticus 5S* has a limited substrate range, being able to ferment fructose, ribose, lactate and glycerol; the latter which may provide an important lipolytic niche for this bacterium in the rumen.

Table 5.1 Least square means of the growth rate of ruminal lipase-producing bacteria during growth in the presence of varying lipid substrates with or without added glucose.

	Mean specific growth rate (μ)[a]					
Energy Substrate	Olive Oil		Corn Oil		Flaxseed Oil	
	Glucose present	Glucose absent	Glucose present	Glucose absent	Glucose present	Glucose absent
A. lipolyticus 5S	0.0752^{ab}	0.0682^{ab}	0.0829	0.0331^{b}	0.0726^{b}	0.0588^{ab}
B. fibrisolvens 49	0.0619^{b}	0.0217^{b}	0.0744	0.0216^{b}	0.0342^{c}	0.0319^{b}
P. avidum	0.0971^{a}	0.0657^{ab}	0.0526	0.0542^{a}	0.0895^{b}	0.0625^{ab}
P. acnes	0.0905^{a}	0.0791^{a}	0.0707	0.0539^{a}	0.1100^{a}	0.0843^{a}
Bacteria	$P = 0.0114$	$P = 0.0311$	$P = 0.0886$	$P = 0.0001$	$P < 0.0001$	$P = 0.0104$
SEM	0.0059	0.0114	0.0072	0.0029	0.0053	0.0440

[a] Least square means within columns with unlike superscripts differ ($P < 0.05$).

Table 5.2 Least square means of the maximum absorbance (A600 nm) ruminal lipase-producing bacteria during growth in the presence of varying lipid substrates with or without added glucose.

	Maximum absorbance (A600 nm)[a]					
Energy Substrate	Olive Oil		Corn Oil		Flaxseed Oil	
	Glucose present	Glucose absent	Glucose present	Glucose absent	Glucose present	Glucose absent
A. lipolyticus 5S	1.3767^{b}	0.9483^{bc}	1.3300^{ab}	1.1433^{b}	1.2300^{bc}	1.1367^{b}
B. fibrisolvens 49	1.3733^{b}	1.1883^{b}	1.3100^{bc}	0.9967^{b}	1.3533^{ab}	1.1117^{b}
P. avidum	1.6400^{a}	1.8500^{a}	1.4567^{a}	1.6000^{a}	1.4933^{a}	1.6800^{a}
P. acnes	1.0117^{c}	0.8933^{c}	1.1767^{c}	0.7657^{b}	1.1300^{c}	0.8653^{c}
Bacteria	$P < 0.0001$	$P < 0.0001$	$P = 0.0012$	$P = 0.0015$	$P = 0.0008$	$P < 0.0001$
SEM	0.0339	0.0634	0.0295	0.0935	0.0420	0.0343

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

The growth rate and maximum growth for *A. lipolyticus* displayed results that fell in about the center of the other results when compared to the other bacteria.

Even though glycerol was used as a negative control through this study, growth curves were performed on each bacterium in the presence of glycerol with or without glucose (Table 5.3). The results indicated that *P. avidum* had the most rapid ($P < 0.05$) rate of growth in the presence of glucose while *P. acnes* had the most rapid ($P < 0.05$) rate of growth in the absence of glucose.

Energy Substrate Comparative Assay with Glucose. Results (Figure 5.1) indicated that *P. avidum* had the highest ($P < 0.05$) rate of FFA accumulation (nmol/mL per h) no matter the substrate when compared to the other bacteria. Flaxseed and olive oil resulted in the highest ($P < 0.05$) rates of FFA accumulation among all four bacterial organisms ($P < 0.05$).

The present study did not show significant interactions between log or stationary phases of growth for lipolytic activity. Results from this study give no indication on when the lipase for these bacteria are potentially produced. A study of *A. lipolyticus* done by Hobson and Summers (1967) suggested the organism produced two enzymes, an esterase associated mainly with the cells and a lipase which is secreted into the culture medium. Results from their study showed that one enzyme was preferentially produced during log phase growth and the other during stationary phase.

Table 5.3 Least square means of the mean specific growth rate and maximum observed absorbance (A600 nm) of ruminal lipase-producing bacteria during growth in the presence of glycerol with or without added glucose.

	Mean specific growth rate (μ)[a]		Maximum absorbance (A600 nm)[a]	
	Glucose present	Glucose Absent	Glucose Present	Glucose Absent
A. lipolyticus 5S	0.0451^{bc}	0.0194^{b}	1.9900^{a}	1.9000^{ab}
B. fibrisolvens 49	0.0265^{c}	0.0145^{b}	1.7767^{a}	1.7267^{ab}
P. avidum	0.1023^{a}	0.0220^{b}	1.9500^{a}	1.9990^{a}
P. acnes	0.0631^{b}	0.0879^{a}	1.5167^{b}	1.3500^{b}
Bacteria	$P = 0.0001$	$P < 0.0001$	$P = 0.0023$	$P = 0.0236$
SEM	0.0065	0.0435	0.0670	0.1399

[a] Least square means within columns with unlike superscripts differ ($P < 0.05$).

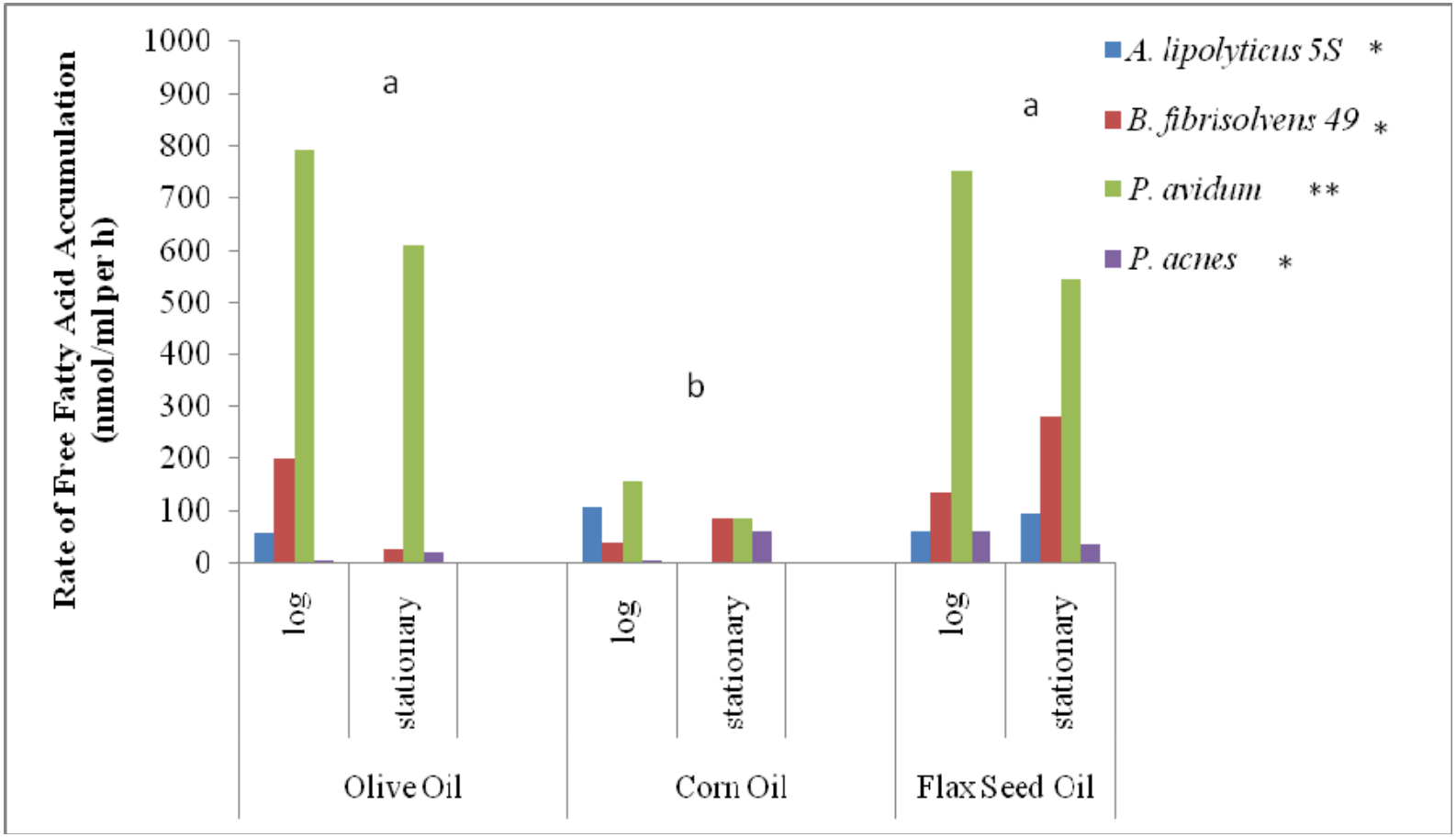

Figure 5.1. Rate of free fatty acid accumulation by pure cultures of ruminal bacteria incubated at 39°C in standard anaerobic medium containing glucose and in the presence of varying added energy substrates. Cultures were stopped at different phases of growth as indicated. [a] Free fatty acid accumulation for each energy substrate with unlike superscripts differ ($P < 0.05$). *Indicates difference in rate of free fatty acid accumulation between each bacteria ($P < 0.05$).

In batch culture, numerous blebs are associated with the walls of *A. lipolyticus* during of early logarithmic phase of growth when lipase production appeared to be maximal (Henderson, 1971; Henderson and Hodgkiss, 1973).

Hazelwood et al. (1979) found that *B. fibrisolvens* almost completely hydrolyzed galactolipids and phospholipids after 8 h of culturing, suggesting the effective lipases were also produced during log growth. However, very little has been reported on the expression of lipase activity against triacylglycerides by this bacterium.

Pablo et al. (1974) found that lipase activity for *P. acnes* appeared to be expressed during early log growth, but expression was diminished in older cultures. Holland et al. (1979) further investigated lipase production by *P. acnes* and *P. avidum*; results suggested that the effects of energy source on the expression of lipase activity may be strain specific. Lipase expression by *P. avidum* appeared to be suppressed by glucose and both glucose and glycerol suppressed lipase production by *P. acnes* strain 37, but neither substrate appeared to influence expression by *P. acnes* strain PF276. The growth curves from the present study showed that growth in glucose containing medium resulted in a reduction in overall maximum growth for *P. avidum* in the presence of each lipid and a reduction ($P < 0.05$) was seen for glucose and glycerol grown cells as well.

Energy Substrate Comparative Assay without Glucose. Results (Figure 5.2) were consistent with the first assay showing that *P. avidum* had the highest ($P < 0.05$) rate of FFA accumulation, but unlike the first energy substrate assay, this study did not show a difference ($P > 0.05$) among oil treatments. Figure 5.1 shows that the FFA accumulation for olive oil and flaxseed did appear to be higher than for corn oil. Overall

the absence or presence of glucose did not appear to make a discernable difference between the two studies.

Implications

The results from this study indicate that olive oil and flaxseed oil are the energy substrates most susceptible to lipolysis and biohydrogenation in the rumen. Therefore, further research *in vivo* should be done to see if limiting their presence in dietary rations could have a substantial impact at reducing ruminal biohydrogenation. Also the results indicated that *P. avidum* had the highest activity in the presence of all the energy substrates suggesting that the focus needs to be on looking at methods to inhibit *P. avidum*.

Based on the growth curves, completed here, glycerol supported the overall highest cell density for all four bacterial organisms. Further research should be completed to ensure that glycerol is not enriching the lipase-producing bacteria that might eventually adapt to the concentration levels being fed.

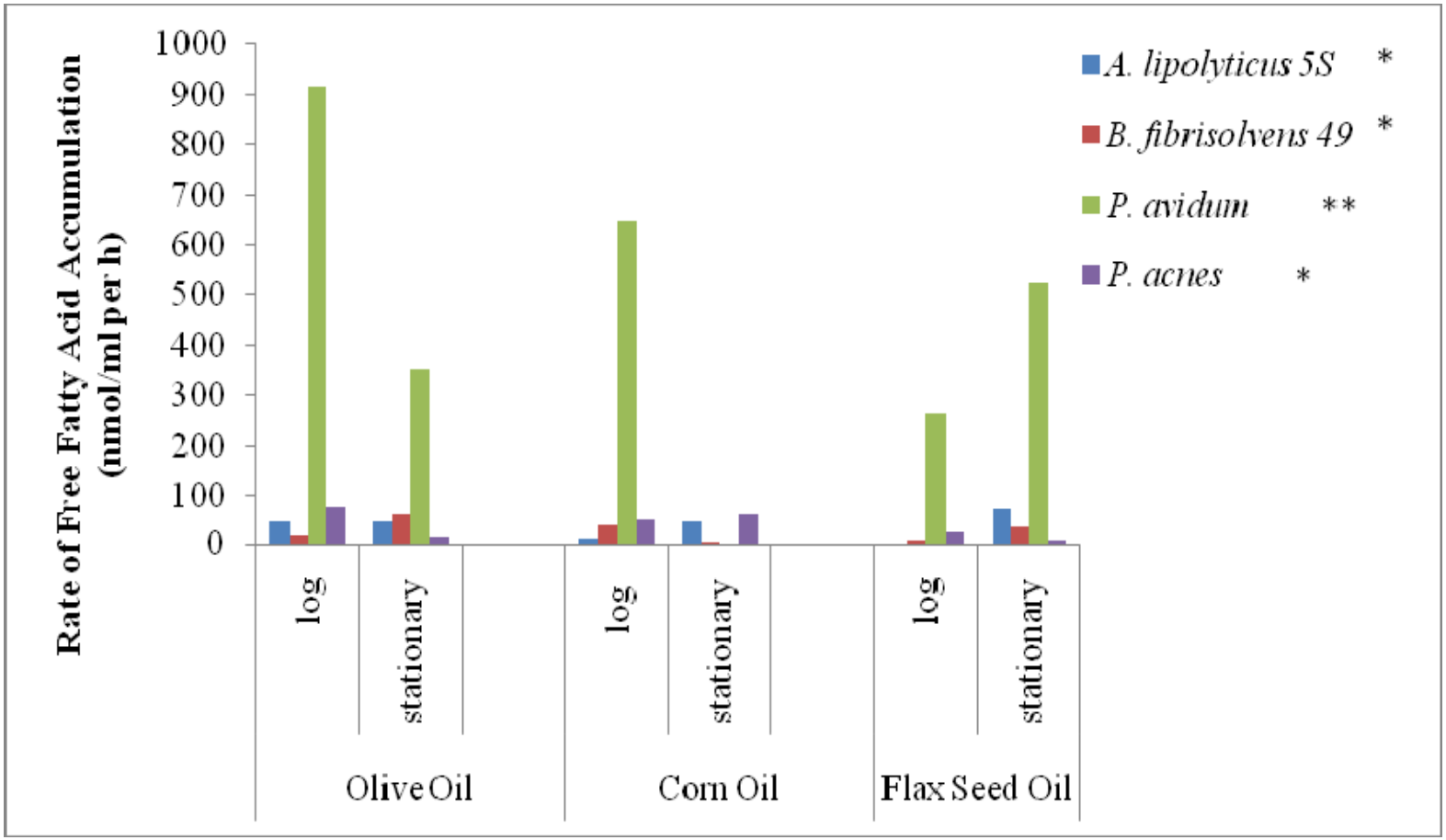

Figure 5.2. Rate of free fatty acid accumulation by pure cultures of ruminal bacteria incubated at 39°C in standard anaerobic medium without glucose and in the presence of varying added energy substrates. Cultures were stopped at different phases of growth as indicated. *Indicates difference in rate of free fatty acid accumulation between each bacteria ($P < 0.05$).

CHAPTER VI

CONCLUSION

Strategies that protect lipids from rumen lipolysis may effectively promote ruminal escape and intestinal absorption of unsaturated fatty acids, resulting in the production of value-added ruminant products enriched with healthy unsaturated fats. Numerous studies have been conducted to characterize the biological and physical factors affecting ruminal lipolysis using rumen contents as incubation materials that likely acted as a solid support to stabilize the hydrophobic-hydrophilic interface, a phenomenon referred to as interfacial activation. A major limitation of studies conducted with particulate matter and digesta is that these materials are not homogenous in size or microbial composition which can lead to considerable variation and experimental error during their use in incubations.

In an attempt to develop a consistent incubation system for culturing ruminal bacteria glass beads were examined as a substitute for rumen contents. Results showed that the introduction of glass beads into an *in vitro* incubation system markedly increased lipolysis activity by establishing a better interface between the water based media and the lipid energy substrate. The use of glass beads during incubation allows for a more controlled system that is uniform and consistent during culturing. In combination to the introduction of glass beads, the use of different head space gasses was also investigated to determine if their use had any effect on ruminal bacteria lipolysis. Previous work has implicated CO_2 as having an inhibitory effect on lipase-producing bacteria. From the

results in this study it was shown that CO_2 did not demonstrate an inhibitory effect. The application of CO_2 into the incubation system allowed for the production of comparable results to other similar studies that traditionally have used CO_2 to establish anaerobic conditions. The development of an *in vitro* ruminal bacterial incubation system will allow us to enrich, isolate and study lipolytic bacteria from the rumen, in hopes that a method to inhibit ruminal lipolysis can be developed.

Previous research has indicated that supplementing glycerol into ruminal diets can inhibit lipolysis up to 60-80%. Studies were conducted to further examine glycerol's effect on ruminal bacteria. Results showed that there does not appear to be a significant justification for supplementing glycerol above 6% concentration in the ruminal diet which, based on other glycerol research, should avoid the possibility of negative effects on neutral detergent fiber digestion. Further research needs to be conducted in order for the best application in feeding increasing amounts of glycerol to be developed to achieve the maximum reduction in the accumulation of free fatty acids without reducing neutral detergent fiber digestion.

This study also focused on pure culture ruminal bacteria in order to provide insight on the mechanism of inhibition that glycerol has on individual bacteria found in the rumen. Results indicated that lipolysis by *B. fibrisolvens 49* and *P. acnes* was not inhibited by end-product inhibition and that it was most likely inhibited by the interruption of the transcription process of mRNA. This knowledge helps us to know that the lipase of *A. lipolyticus 5S* and *P. avidum* is most likely easier to manipulate and

inhibit. Therefore, our attention is more directed on trying to inhibit the lipase activity of *A. lipolyticus 5S* and *P. avidum* versus the other experimental bacteria.

In addition, the effects of introducing different energy substrates in the presence of pure cultures of ruminal bacteria were evaluated to better characterize the effects of those substrates on the lipid profile most likely found in the rumen. Olive oil and flaxseed oil appeared to be the most susceptible energy substrates to lipolysis in this study. It is possible the bacteria targeted these two energy sources more than corn oil due to their fatty acid composition. Olive oil is composed primarily of oleic acid, flaxseed oil is composed mainly of α-linolenic, acid and corn oil consists of predominantly linoleic acid. Therefore, this work supports supplementing more corn oil in the ruminal diet due to its potential to escape the majority of lipolysis.

The ruminal bacterium *P. avidum* consistently demonstrated across every study to have the highest amount of lipolysis activity indicating that the high rate of lipolysis in the rumen could be contributed to mainly *P. avidum*. The combination of these studies suggest that further work needs to be done in the direction of developing a strategy for inhibiting *P. avidum* with the overall goal of protecting rumen susceptible fatty acids such as oleic acid and flaxseed oil in an attempt to reduce lipolysis and biohydrogenation.

LITERATURE CITED

Albert, C. M., H. Campos, M. J. Stampfer, P. M. Ridker, J. E. Manson et al. 2002. Blood levels of long-chain n–3 fatty acids and the risk of sudden death. New Engl. J. Med. 346:1113-1118.

Ashes, J., B. Siebert, S. Gulati, A. Cuthbertson, and T. Scott. 1992. Incorporation of n−3 fatty acids of fish oil into tissue and serum lipids of ruminants. Lipids 27:629-631.

Bauman, D. E., L. H. Baumgard, B. A. Corl, and J. M. Griinari. 2000. Biosynthesis of conjugated linoleic acid in ruminants. J. Anim. Sci. 77:1-15.

Baumgard, L. H., E. Matitashvili, B. A. Corl, D. A. Dwyer, and D. E. Bauman. 2002. Trans-10, cis-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. J. Dairy Sci. 85:2155-2163.

Baylin, A., E. K. Kabagambe, A. Ascherio, D. Spiegelman, and H. Campos. 2003. Adipose tissue {alpha}-linolenic acid and nonfatal acute myocardial infarction in costa rica. Circulation 107:1586-1591.

Brown, D. W., and W. E. C. Moore. 1960. Distribution of *Butyrivibrio fibrisolvens* in nature. J. Dairy Sci. 43:1570-1574.

Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. 25:1324-1328.

Bryant, M. P., and N. Small. 1956. The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol. 72:16-21.

Cheng, K. J., and J. W. Costerton. 1977. Ultrastructure of *Butyrivibrio fibrisolvens*: a Gram-positive bacterium. J. Bacteriol. 129:1506-1512.

Chopra, I., and K. Hacker. 1989. Effects of tetracyclines on the production of extracellular proteins by members of the propionibacteriaceae. FEMS Microb. Lett. 60:21-24.

Chopra, I., and A. Linton. 1987. The antibacterial effects of low concentrations of antibiotics. p 211-259. In Advances in Microbial Physiology. A. H. Rose and D. W. Tempest (eds.) 28. Academic Press.

Chow, T. T., V. Fievez, A. P. Moloney, K. Raes, D. Demeyer et al. 2004. Effect of fish oil on in vitro rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. Anim. Feed Sci. Technol. 117:1-12.

Cirne, D. G., O. D. Delgado, S. Marichamy, and B. Mattiasson. 2006. *Clostridium lundense* sp. nov., a novel anaerobic lipolytic bacterium isolated from bovine rumen. Int. J. Syst. Evol. Microbiol. 56:625-628.

Clarke, D. G., and J. C. Hawke. 1970. Studies on rumen metabolism VI. —in vitro hydrolysis of triglyceride and isolation of a lipolytic fraction. J. Sci. Food Agric. 21:446-452.

Cotta, M. A., and R. B. Hespell. 1986. Proteolytic activity of the ruminal bacterium *Butyrivibrio fibrisolvens*. Appl. Environ. Microbiol. 52:51-58.

Cotta, M. A., and J. B. Russell. 1982. Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. J. Dairy Sci. 65:226-234.

Cove, J. H., K. T. Holland, and W. J. Cunliffe. 1983. Effects of oxygen concentration on biomass production, maximum specific growth rate and extracellular enzyme production by three species of cutaneous Propionibacteria grown in continuous culture. J. Gen. Microbiol. 129:3327-3334.

Cummins, C. S., and J. L. Johnson. 1974. *Corynebacterium parvum*: a synonym for *Propionibacterium acnes*? J. Gen. Microbiol. 80:433-442.

Dalrymple, B. P., Y. Swadling, I. Layton, K. S. Gobius, and G.-P. Xue. 1999. Distribution and evolution of the xylanase genes xynA and xynB and their homologues in strains of *Butyrivibrio fibrisolvens*. Appl. Environ. Microbiol. 65:3660-3667.

de Veth, M. J., S. K. Gulati, N. D. Luchini, and D. E. Bauman. 2005. Comparison of calcium salts and formaldehyde-protected conjugated linoleic acid in inducing milk fat depression. J. Dairy Sci. 88:1685-1693.

Delbecchi, L., C. E. Ahnadi, J. J. Kennelly, and P. Lacasse. 2001. Milk fatty acid composition and mammary lipid metabolism in holstein cows fed protected or unprotected canola seeds. J. Dairy Sci. 84:1375-1381.

Dietschy, J. M., L. A. Woollett, and D. K. Spady. 1993. The interaction of dietary cholesterol and specific fatty acids in the regulation of LDL receptor activity and plasma LDL-cholesterol concentrationsa. Annals N.Y. Acad. Sci. 676:11-26.

Dighe, A. S., Y. S. Shouche, and D. R. Ranade. 1998. *Selenomonas lipolytica* sp. nov., an obligately anaerobic bacterium possessing lipolytic activity. Int. J. Syst. Bacteriol. 48:783-791.

Dohme, F., V. Fievez, K. Raes, and D. I. Demeyer. 2003. Increasing levels of two different fish oils lower ruminal biohydrogenation of eicosapentaenoic and docosahexaenoic acid in vitro. Anim. Res. 52:309-320.

Doreau, M., and Y. Chilliard. 1997. Digestion and metabolism of dietary fat in farm animals. Br. J. Nutr. 78:S15-S35.

Emken, E. 1995. *Trans* fatty acids and coronary heart disease risk. Physiochemcial properties,
intake, and metabolism. Am. J. Clin. Nutr.:659S-669S.

Fadíloglu, S., and O. Erkmen. 2002. Inactivation of lipase by carbon dioxide under atmospheric pressure. J. Food Eng. 52:331-335.

Faichney, G. J., T. W. Scott, and L. J. Cook. 1973. The utilization by growing lambs of a casein-safflower oil supplement treated with formaldehyde. Aust. J. Biol. Sci. 26:1179

Garrett, W. N., Y. T. Yang, W. L. Dunkley, and L. M. Smith. 1976. Increasing the polyunsaturated fat content of beef and lamb. J. Anim. Sci. 42:845-853.

Garton, G. A., P. N. Hobson, and A. K. Lough. 1958. Lipolysis in the rumen. Nature 182:1511-1512.

Garton, G. A., A. K. Lough, and E. Vioque. 1961. Glyceride hydrolysis and glycerol fermentation by sheep rumen contents. J. Gen. Microbiol. 25:215-225.

Gebauer, S. K., T. L. Psota, W. S. Harris, and P. M. Kris-Etherton. 2006. n-3 Fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. Am. J. Clin. Nutr. 83:S1526-1535.

Gilbery, T. C., G. P. Lardy, and M. L. Bauer. 2010. Effects of glycerol supplementation on dry matter intake, rate and site of digestion, and ruminal ferementation in cannulated steers fed finishing diets., NDSU ND Agricultural Experiment Station.

Greenman, J., K. T. Holland, and W. J. Cunliffe. 1983. Effects of pH on biomass, maximum specific growth rate and extracellular enzyme production by three species of cutaneous Propionibacteria grown in continuous culture. J. Gen. Microbiol. 129:1301-1307.

Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist et al. 1998. *Trans*-octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci. 81:1251-1261.

Gulati, S. K., T. W. Scott, and J. R. Ashes. 1997. In-vitro assessment of fat supplements for ruminants. Anim. Feed Sci. Technol. 64:127-132.

Harfoot, C., R. Noble, and J. Moore. 1975. The role of plant particles, bacteria and cell-free supernatant fractions of rumen contents in the hydrolysis of trilinolein and the subsequent hydrogenation of linoleic acid. Antonie van Leeuwenhoek 41:533-542.

Harfoot, C. G., and G. P. Hazlewood. 1997. Lipid metabolism in the rumen. In: P. N. Hobson and C. S. Stewart (eds.). Blackie Academic & Professional, New York.

Harfoot, C. G., R. C. Noble, and J. H. Moore. 1973. Factors influencing the extent of biohydrogenation of linoleic acid by rumen micro-organisms in vitro. J. Sci. Food Agric. 24:961-970.

Harvatine, K. J., and M. S. Allen. 2006. Fat supplements affect fractional rates of ruminal fatty acid biohydrogenation and passage in dairy cows. J. Nutr. 136:677-685.

Hawke, J. C., and W. R. Silcock. 1970. The in vitro rates of lipolysis and biohydrogenation in rumen contents. Biochim. Biophys. 218:201-212.

Hayes, K. C. 2000. Dietary fatty acids, cholesterol, and the lipoprotein profile. Br. J. Nutr. 84:397-399.

Hazlewood, G. P., D. G. Clarke, and R. M. C. Dawson. 1980. Complex lipids of a lipolytic and general-fatty -acid-requiring *Butyrivibrio* sp. isolated from the ovine rumen. Biochem J. 191:555-560.

Hazlewood, G. P., M. J. Reynolds, R. M. C. Dawson, and F. D. Gunstone. 1979. An automatic colorimeter and its use in evaluating the growth response of an anaerobic general fatty acid auxotroph to cis- and trans-octadecenoic acids. J. Appl. Microbiol. 47:321-325.

Henderson, C. 1971. A study of the lipase produced by *Anaerovibrio lipolytica*, a rumen bacterium. J. Gen. Microbiol. 65:81-89.

Henderson, C. 1973. An Improved method for enumerating and isolating lipolytic rumen baceria. J. Appl. Microbiol. 36:187-188.

Henderson, C. 1975. The isolation and characterization of strains of lipolytic bacteria from the ovine rumen. J. Appl. Microbiol. 39:101-109.

Henderson, C., and W. Hodgkiss. 1973. An electron microscopic study of *Anaerovibrio lipolytica* (strain 5s) and its lipolytic enzyme. J. Gen. Microbiol. 76:389-393.

Hespell, R. B., K. Kato, and J. W. Costerton. 1993. Characterization of the cell wall of *Butyrivibrio* species. Can. J. Microbiol. 54:912-921.

Hespell, R. B., and P. J. O'Bryan-Shah. 1988. Esterase activities in *Butyrivibrio fibrisolvens* strains. Appl. Environ. Microbiol. 54:1917-1922.

Hirashima, A., G. Childs, and M. Inouye. 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. 79:373-389.

Hobson, P. N., and S. O. Mann. 1961. The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of the sheep. J. Gen. Microbiol. 25:227-240.

Hobson, P. N., and R. Summers. 1967. The continuous culture of anaerobic bacteria. J. Gen. Microbiol. 47:53-65.

Hogan, J., and R. Hogan. 1976. The evaluation of formaldehyde-treated sunflower seed-casein supplement as a source of linoleic acid for ruminant lipids. Aust. J. Agri. Res. 27:129-138.

Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe laboratory manual, 4th edn. Virginia Polytechnic Institute and State University, Blacksburg, VA.

Holland, K. T., J. Greenman, and W. J. Cunliffe. 1979. Growth of cutaneous Propionibacteria on synthetic medium; growth yields and exoenzyme production. J. Appl. Microbiol. 47:383-394.

Hu, F. B., M. J. Stampfer, J. E. Manson, E. B. Rimm, A. Wolk et al. 1999. Dietary intake of α-linolenic acid and risk of fatal ischemic heart disease among women. The American Journal of Clinical Nutrition 69:890-897.

Ingham, E., K. T. Holland, G. Gowland, and W. J. Cunliffe. 1981. Partial purification and characterization of lipase (EC 3.1.1.3) from *Propionibacterium acnes*. J. Gen. Microbiol. 124:393-401.

Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel et al. 1994. Bacterial lipases. FEMS Microbiol. Rev. 15:29-63.

Jarvis, G. N., and E. R. B. Moore. 2010. Lipid metabolism and the rumen microbial ecosystem. p 2245-2257. In Handbook of Hydrocarbon and Lipid Microbiology. K. N. Timmis (ed.). Springer Berlin Heidelberg.

Jarvis, G. N., C. Strompl, E. R. Moore, and J. H. Thiele. 1998. Isolation and characterisation of obligatory anaerobic, lipolytic bacteria from the rumen of red deer. Syst. Appl. Microbiol. 21:135-143.

Jarvis, G. N., C. Strömpl, E. R. B. Moore, and J. H. Thiele. 1999. Isolation and characterization of two glycerol-fermenting clostridial strains from a pilot scale anaerobic digester treating high lipid-content slaughterhouse waste. J. Appl. Microbiol. 86:412-420.

Jenkins, T. C., and W. C. Bridges. 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. Eur. J. Lipid Sci. Technol. 109:778-789.

Kemp, P., D. J. Lander, and R. T. Holman. 1984. The hydrogenation of the series of methylene-interrupted cis,cis-octadecadienoic acids by pure cultures of six rumen bacteria. Br. J. Nutr. 52:171-177.

Kepler, C. R., K. P. Hirons, J. J. McNeill, and S. B. Tove. 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. J. Biol. Chem. 241:1350-1354.

Kerley, M. C. 2007. Could glycerin - a biodiesel byproduct - be used as a cattle feed? Sci. Daily. http://www.sciencedaily.com/releases/2007/05/070525090245.htm Accessed Date Accessed.| doi:DOI|

Koch, A. L. 1981. Growth measurement. p 179-207. In Manual of methods for general bacteriology. P. Gerhardt et al. (eds.). American Society for Microbiology, Washington DC.

Krueger, N. A., R. C. Anderson, T. R. Callaway, T. S. Edrington, and D. J. Nisbet. 2008. Isolation of prominent lipolytic rumen bacteria. J. Anim Sci. 86, E-Suppl. 2/J. Dairy Sci., Vol. 91, E. Suppl. 1:87.

Krueger, N. A., R. C. Anderson, L. O. Tedeschi, T. R. Callaway, T. S. Edrington et al. 2010. Evaluation of feeding glycerol on free-fatty acid production and fermentation kinetics of mixed ruminal microbes in vitro. Bior. Technol. 101:8469-8472.

Kwon, D., and J. Rhee. 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. J. Am. Oil Chem. Soc. 63:89-92.

Latham, M. J., J. E. Storry, and M. E. Sharpe. 1972. Effect of low-roughage diets on the microflora and lipid metabolism in the rumen. Appl. Environ. Microbiol. 24:871-877.

Lee, C. Y., and J. J. Iandolo. 1986. Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a genome into the lipase structural gene. J. Bacteriol. 166:385-391.

Leigh-Firbank, E. C., A. M. Minihane, D. S. Leake, J. W. Wright, M. C. Murphy et al. 2002. Eicosapentaenoic acid and docosahexaenoic acid from fish oils: differential associations with lipid responses. Br. J. Nutr. 87:435-445.

Leyendecker, S. A., T. R. Callaway, R. C. Anderson, and D. J. Nisbet. 2004. Technical note on a much simplified method for collecting ruminal fluid using a nylon paint strainer. J. Sci. Food Agri. 84:387-389.

Lourenço, M., E. Ramos-Morales, and R. J. Wallace. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. Anim. 4:1008-1023.

Magasanik, B. 1961. Cellular regulatory mechanism. Cold Spring Harbor Symposium on Quantive Biology:249-256.

Maia, M., L. Chaudhary, C. Bestwick, A. Richardson, N. McKain et al. 2010. Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. BMC Microbiol. 10:52.

Martinez, D. A., and B. C. Nudel. 2002. The improvement of lipase secretion and stability by addition of inert compounds into *Acinetobacter calcoaceticus* cultures. Can. J. Microbiol. 48:1056-1061.

Maruyama, T., M. Nakajima, S. Uchikawa, H. Nabetani, S. Furusaki et al. 2000. Oil-water interfacial activation of lipase for interesterification of triglyceride and fatty acid. J. Am. Oil Chem. Soc. 77:1121-1127.

McInerey, M. 1988. Anaerobic hydrolysis and fermentation of fats and proteins. In: A. Zehnder (ed.) In biology of anaerobic microorganisms.p 373-414. John Wiley and Sons, New York.

Medicine, I. 2002. Dietary reference intakes, part I.p 8-1-8-97 National Academies Press.

Mensink, R. P., P. L. Zock, A. D. Kester, and M. B. Katan. 2003. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am. J. Clin. Nutr. 77:1146-1155.

Miskin, J. E., A. M. Farrell, W. J. Cunliffe, and K. T. Holland. 1997. *Propionibacterium acnes*, a resident of lipid-rich human skin, produces a 33 kDa extracellular lipase encoded by *gehA*. J. Microbiol. 143:1745-1755.

Morris, D. 2008a. Adding linseed to feed enhances the fat profile of beef. Linseed in the ruminant diet. Flax Council of Cananda, Winnipeg, MB

Morris, D. 2008b. Food sources of alpha-linolenic acid. New Flax Facts. Flax Council of Canada, Winnipeg, MB.

Mosley, E., G. Powell, M. Riley, and T. Jenkins. 2002. Microbial biohydrogenation of oleic acid to *trans* isomers *in vitro*. J. Lipid Res. 43:290-296

Pablo, G., A. Hammons, S. Bradley, and J. E. Fulton, Jr. 1974. Characteristics of the extracellular lipase from *Corynebacterium acnes* and *Staphylococcus epidermis*. J. Invest. Dermatol. 63:231-238.

Perfield II, J. W., A. L. Lock, A. M. Pfeiffer, and D. E. Bauman. 2004. Effects of amide-protected and lipid-encapsulated conjugated linoleic acid (CLA) supplements on milk fat synthesis. J. Dairy Sci. 87:3010-3016.

Piovant, M., S. Varenne, J. M. Pagès, and C. Lazdunski. 1978. Preferential sensitivity of syntheses of exported proteins to translation inhibitors of low polarity in *Escherichia coli*. Mol. Gen. Genetics 164:265-274.

Polan, C. E., J. J. McNeill, and S. B. Tove. 1964. Biohydrogenation of unsaturated fatty acids by rumen bacteria. J. Bacteriol. 88:1056-1064.

Pollock, M. R. 1962. Exoenzymes. In: I. C. Gunsalus and R. Y. Stanier (eds.) In The Bacteria No. 4.p 121-178. Academic Press, New York and London.

Prins, R., A. Lankhorst, P. van der Meer, and C. Van Nevel. 1975. Some characteristics of *Anaerovibrio lipolytica*; a rumen lipolytic organism. Antonie van Leeuwenhoek 41:1-11.

Rego, O. A., H. J. D. Rosa, P. V. Portugal, T. Franco, C. M. Vouzela et al. 2005. The effects of supplementation with sunflower and soybean oils on the fatty acid profile of milk fat from grazing dairy cows. Anim. Res. 54:17-24.

Rowe, M. T. 1988. Effect of carbon dioxide on growth and extracellular enzyme production by *Pseudomonas fluorescens B52*. Inter. J. Food Microbiol. 6:51-56.

Sackmann, J. R., S. K. Duckett, M. H. Gillis, C. E. Realini, A. H. Parks et al. 2003. Effects of forage and sunflower oil levels on ruminal biohydrogenation of fatty acids and conjugated linoleic acid formation in beef steers fed finishing diets. J. Anim Sci. 81:3174-3181.

Schifferli, D. M., and E. H. Beachey. 1988. Bacterial adhesion: modulation by antibiotics with primary targets other than protein synthesis. Antimicrob. Agents Chemother. 32:1609-1613.

Shorland, F. B., R. O. Weenink, and A. T. Johns. 1955. Effect of the rumen on dietary fat. Nature 175:1129-1130.

Stack, R. J. 1988. Neutral sugar composition of extracellular polysaccharides produced by strains of *Butyrivibrio fibrisolvens*. Appl. Environ. Microbiol. 54:878-883.

Stackebrandt, E., and K. Schaal. 2006. Family Propionibacteriaceae: The genus *Propionibacterium*. p 383-399. In Prokaryotes. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt (eds.). Springer New York.

Strompl, C., B. J. Tindall, G. N. Jarvis, H. Lunsdorf, E. R. B. Moore et al. 1999. A re-evaluation of the taxonomy of the genus *Anaerovibrio*, with the reclassification of *Anaerovibrio glycerini* as *Anaerosinus glycerini* gen. nov., comb. nov., and *Anaerovibrio burkinabensis* as *Anaeroarcus burkinensis* [corrig.] gen. nov., comb. nov. Int. J. Syst. Bacteriol. 49:1861-1872.

Tymchuk, S. M., G. R. Khorasani, and J. J. Kenelly. 1998. Effect of feeding formaldehyde- and heat-treated oil seed on milk yield and milk composition. Can. J. Anim. Sci. 78:693.

Van Nevel, C., and D. I. Demeyer. 1995. Lipolysis and biohydrogenation of soybean oil in the rumen in vitro: inhibition by antimicrobials. J. Dairy Sci. 78:2797-2806.

Wahrburg, U. 2004. What are the health effects of fat? Eur. J. Nutr. 43:i6-i11.

Wallace, J. R., L. C. Chaudhary, N. McKain, N. R. McEwan, A. J. Richardson et al. 2006. *Clostridium proteoclasticum*: a ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microb. Lett. 265:195-201.

Wasowska, I., M. R. G. Maia, K. M. Nied?wiedzka, M. Czauderna, J. M. C. Ramalho Ribeiro et al. 2006. Influence of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids. Br. J. Nutr. 95:1199-1211.

Weill, P., B. Schmitt, G. Chesneau, N. Daniel, F. Safraou et al. 2002. Effects of introducing linseed in livestock diet on blood fatty acid composition of consumers of animal products. Annals Nutr. Metabol. 46:182-191.

Weimer, P. J. 1998. Manipulating ruminal fermentation: a microbial ecological perspective. J. Anim Sci. 76:3114-3122.

Wijendran, V., and K. C. Hayes. 2004. Dietary n-6 and n-3 fatty acid balance and cardiovascular health. Ann. Rev. Nutr. 24:597-615.

Woollett, L. A., D. K. Spady, and J. M. Dietschy. 1992. Regulatory effects of the saturated fatty acids 6:0 through 18:0 on hepatic low density lipoprotein receptor activity in the hamster. J. Clin. Invest. 89:1133-1141.

Zimmer, Z. 1999. An evaluation of the ruminal establishment of *Proprionibacterium* species in dairy cattle using three different treatment. Direct fed microbials for the millenium and beyond. Bio Vet Inc., Blue Mounds, WI.

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