ABSTRACT

JOHNSON, MEREDITH. Understanding Rumen Fermentation: I. Effect of High DHA Algal Oil on Microbial Biohydrogenation and II. Monitoring Microbial Shifts in Response to Antibiotics and Oil using T-RFLP Analysis. (Under the direction of Dr. Vivek Fellner.)

Two experiments were conducted to study rumen fermentation in continuous and batch culture. The objective of the first study was to investigate the potential of marine algae to enhance incorporation of long-chain polyunsaturated fatty acids (LC-PUFA) into milk. Consumption of LC-PUFA has proven to improve human health by reducing the risk of heart disease, obesity, and diabetes, and their incorporation into milk would provide a way to increase omega-3 (n-3) consumption. Mixed cultures of rumen microbes were incubated in artificial fermentors for a total of 9d. Each run consisted of 6 fermentors. The fermentors were gradually adapted over 4 d to a diet with a 50:50 forage:concentrate ratio from an all-forage diet. One fermentor served as a control (C) and only received the basal diet of corn silage and concentrate mix. Two fermentors served as treatment and received the basal diet plus supplemental algal oil. One received the oil in its extracted (E) form while another received it intact (I). Fermentation variables measured were volatile fatty acids (VFA), methane production, culture pH, ammonia (NH_3), and long-chain fatty acids (LCFA). Compared to C, addition of the algal oil tended to lower acetate ($p<0.10$), increase propionate ($p<0.03$), and decrease the A:P ratio ($p<0.03$). The algal oil also lowered methane production ($p<0.002$), and raised culture pH ($p<0.001$). There were differences in VFA production between E and I, as I had lower acetate ($p<0.05$), higher propionate ($p<0.005$), and lower A:P ($p<0.01$) than E. Methane production ($p<0.001$) and culture pH ($p<0.03$) were lower in I than E. Control cultures had significantly greater C18:0 when compared with cultures receiving the algal

oil. Algal oil, either intact or extracted, resulted in substantial quantities of LC-PUFA in ruminal cultures reflecting the greater concentrations of those fatty acids in the oil. Biohydrogenation was greater in C when compared to either E or I but the concentration of 18:2 was similar across all treatments. A second in vitro batch culture study was conducted to monitor the effects of the C, E, and I diets on fatty acid biohydrogenation over time. A 0.5 g substrate was placed into 200 ml bottles and 30 ml of a mixture of rumen inoculum and artificial saliva (2:1 ratio) were added. Bottles were capped and placed in a water bath at 39^{o}C for 0, 2, 4, 6, and 24hr. Three bottles per treatment were used to provide for additional replication (n=3). The differences in the percentages of the fatty acids across treatments at 24hr were similar to those found in the continuous culture study. However, the quantities of the fatty acids revealed the same amount of C18:2 across treatments with increased *cis*-C18:1 and *trans*-C18:1 in the E and I. Little biohydrogenation of the LC-PUFA was observed, and DHA decreased by an average of 2.7 mg. Our data suggest that biohydrogenation of the LC-PUFA in the algal oil was not extensive. There was an accumulation of the 18:1 *cis* and *trans* isomers in cultures receiving E and I and it seems that the DHA and DPA in the algal oil interfered with the terminal step of 18:1 biohydrogenation to 18:0.

The objective of the second study was to investigate microbial population changes in response to the antibiotics monensin (M) and bacitracin (B) and supplemental fat in the form of oil (O). Their metabolic effects are similar and well known, but it is not clear how they alter the microbial populations in the rumen to achieve these affects. The objective of this study was to investigate microbial population changes in response to the antibiotics monensin (M) and bacitracin (B) and supplemental fat in the form of oil (O).

Mixed cultures of rumen microbes were incubated in artificial fermentors for a total of 16 d. Each run consisted of 6 fermentors. One served as a control (C) and received alfalfa hay, while the other four received one of the following four treatments; 1) monensin then oil (MO), 2) oil then monensin (OM), 3) bacitracin then oil (BO) and, 4) oil then bacitracin (OB). Each run was replicated three times (n=3). Fermentation variables measured were volatile fatty acids (VFA), total cell number, methane production, culture pH, ammonia (NH_3), and long-chain fatty acids (LCFA). Samples were also taken to analyze the microbial population using terminal restriction fragment length polymorphisms (T-RFLP).

There were no significant changes in the fermentation parameters in C. As expected, both M and O reduced acetate ($p<0.01$), increased propionate ($p<0.05$), and decreased methane production ($p<0.05$). Bacitracin did not alter acetate or propionate but reduced methane ($p<0.05$). The sequence of additive supplementation did not alter rumen fermentation, but it did seem to cause differences in the microbial populations. The T-RFLP of C showed an adaptation to the in vitro system before d4, indicating a decrease in diversity occurring between d10 and d16. There were differences between M and B in diversity and in divisions at the Class level, reflecting the different mode of action of the two antibiotics. M and O also were different from each other, suggesting the additives affect different microorganisms. Furthermore, the sequences in which the additives were supplemented affected the microbial populations.

UNDERSTANDING RUMEN FERMENTATION:

I. EFFECT OF HIGH DHA ALGAL OIL ON MICROBIAL BIOHYDROGENATION AND II. MONITORING MICROBIAL SHIFTS IN RESPONSE TO ANTIBIOTICS AND OIL USING T-RFLP ANALYSIS.

by

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DEDICATION

This thesis is dedicated in loving memory to my father, Franklin Johnson, and to his sister, Alicia Holder. Their devotion to their loved ones, infectious sense of humor, and unfailing dignity and strength in the face of hardship are qualities I can only hope to attain in my lifetime.

BIOGRAPHY

Meredith Christina Johnson was born on November 29, 1982 to Marie and Franklin Johnson. She grew up in Cameron, NC with her two brothers, Don and Kevin. In high school, she was very active in band, swimming, and various clubs and community service activities. She was the valedictorian of Lee County Senior High School's class of 2001.

That spring, Meredith received an NC State Merit Recognition Scholarship and enrolled in the University in the fall of 2001. She remained involved in music and was also a member of the NCSU Crew Team. She was an active participant in the University Scholars Program and served as an Undergraduate Teaching Assistant for three years. After receiving a summer research grant through the Honors Program, she conducted research under Dr. Theo van Kempen. She worked in Dr. Jorge Piedrahita's laboratory and assisted with embryo transfer surgeries for two years. She was president of the NCSU's chapter of the Golden Key International Honor Society and was inducted into Phi Kappi Phi. Meredith completed the CALS honors program, earned a BS in Animal Science and minors in nutrition and genetics, and graduated Summa Cum Laude in May of 2005.

Meredith received an assistantship from the graduate school and began her masters program in the fall of 2005. She conducted her graduate research, which focused on the microbial population in the rumen, under Dr. Vivek Fellner. Her *in vitro* experiments focused on DHA supplementation and the effects of oil and antibiotics on the rumen. Meredith hopes to continue working in a research environment throughout her career.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Supplemental fat research began with studying how to meet the energy requirements of lactating cows, and the data from those studies as well as more recent ones has helped research on improving milk fat composition (Jenkins and McGuire, 2006). Recent research is primarily aimed at improving interactions between fatty acids and microbes to reduce antimicrobial effects while controlling microbial biohydrogenation and thus producing healthier milk fat (Jenkins, 1993). Along with supplemental fat, animals in high production, such as lactating or feedlot cattle, are often fed ionophores such as monensin to help meet their high demand for energy. Both fat and ionophores alter rumen fermentation, but it is not clear how they alter the microbial populations in the rumen to achieve these effects.

Much research has been aimed at improving the fatty acid profile of milk so that the proportion of saturated fat is decreased and that of unsaturated fatty acids is increased (Jenkins and McGuire, 2006). Recent efforts have focused on increasing the concentration of LC-PUFA, particularly that of omega-3 (n-3) FA, in milk. These fatty acids have been shown to enhance infant growth and visual and cognitive development and reduce the risk of cardiovascular disease, autoimmune disorders, type 2 diabetes, hypertension, rheumatoid arthritis, and certain cancers (Connor, 2000, Simopoulos, 1999).

In spite of these health benefits, intake of n-3 fatty acids from typical diets is usually low and the n-6 intake is high. This raises the n-6 to n-3 ratio to about 20-30:1, up from the healthier 1-4:1 (Simopoulos, 1999). The skewed n-6 to n-3 ratio perpetuates metabolic disorders by increasing pro-inflammatory products, therefore promoting

thrombi and atheromas formation, leading to allergic and inflammatory disorders, and contributing to cell proliferation. Increased n-3, on the other hand, produces hypolipidemic, antithrombotic, and anti-inflammatory effects (Simopoulos, 1999).

Besides egg yolk, chicken, and oily fish, human milk and supplemented food are the only foods in the typical US diet that are a good source of DHA. As of 2004, no infant formula or baby foods in the United States contained fish (Hoffman, et al., 2004). Mothers are discouraged from eating too much fish because of the risk of metal contamination and, like children, may consume very little or no fish (Oken, et al., 2003). This makes it imperative to provide sources of n-3 PUFA apart from fish or fish oil supplements. Because of the already beneficial effects of consuming dairy for mothers and children, enrichment of milk with healthful fatty acids would help provide a portion of the recommended daily LC-PUFA allowance.

Numerous factors affect the fate of unsaturated fatty acids in the rumen, and the most extensively studied are those that affect microbial activity or biohydrogenation (Jenkins, 1993). Biohydrogenation occurs after hydrolysis and is a process during which microbial enzymes add hydrogens to unsaturated fatty acids, making them more saturated (Jenkins and McGuire, 2006). Extensive biohydrogenation of unsaturated fatty acids in the rumen is implicated as a major process that determines the profile of fatty acids supplied to the mammary gland. Thus, strategies to enhance milk fat LC-PUFA would involve increasing rumen outflow of these fatty acids and their subsequent incorporation into milk fat. The fatty acids may have to be delivered to the rumen in a protected form, such as the raw plant material, rather than the extracted oil.

Numerous groups have attempted to use fish oil to increase LC-PUFA in the milk (AbuGhazaleh, et al., 2002, Donovan, et al., 2000, Lock and Bauman, 2004). Several studies have incubated rumen fluid with fish oil or pure LC-PUFA to investigate their fate in the rumen, including their possible effects on and involvement in biohydrogenation (AbuGhazaleh and Jenkins, 2004a, AbuGhazaleh and Jenkins, 2004b, Dohme, et al., 2003, Gulati, et al., 1999). These experiments have shown that both the oil concentration in the fluid and the fatty acid concentration in the oil influence fatty aid metabolism (AbuGhazaleh and Jenkins, 2004a)

In the first study that fed marine algae, there was incorporation of LC-PUFA into the milk, and the algae protected with xylose had even greater incorporation (Franklin, et al., 1999). The objectives of the research outlined in chapter 3 of this thesis were to 1) monitor the changes in rumen fermentation in response to algal oil, including biohydrogenation, and to 2) investigate possible protection from biohydrogenation by the algal oil in its intact form. It was hypothesized that including algal oil in its intact form in contrast to algal oil in its extracted form would impart greater degree of inertness in the rumen and thus allow for greater incorporation into milk fat.

In addition to studying biohydrogenation of supplemental fat in the rumen, it is also important to study the shifting in the microbial populations upon exposure to oil and other supplements. Cows in peak lactation are fed supplemental fat and ionophores to help meet their tremendous metabolic requirements. Individually, these additives increase feed efficiency. Fat or oil (O) provides a more energy dense diet without requiring a fiber reduction (Palmquist and Jenkins, 1980), and ionophores improve the feed:gain ratio (Pressman, 1976). In the United States, monensin (M) has been fed to

beef cattle since 1975, to lactating dairy cattle since 2005, and is the most commonly tested and used ruminant ionophore.

Both O and M improve the fermentation profile, as they decrease acetate:propionate and reduce methane production (Jenkins, 1993, Russell and Strobel, 1989). The effects of O and M on pH are inconsistent (Jenkins, et al., 2003). Because of their ability to alter the fermentation of the rumen, O and M likely have effects on the microbial population (Jenkins, et al., 2003, Sauer, et al., 1998, Van Nevel and Demeyer, 1995). Supplemental fat may physically coat fiber with fat, bind to microbial cells and affect the cell membranes, and may change competition, and thus the populations, in the rumen (Palmquist and Jenkins, 1980). Monensin is most active against Gram (+) bacteria, which lack much of the protective outer membrane that makes Gram (-) bacteria less susceptible to the antibiotic (Russell, 1987). Monensin is an antiporter that disrupts the ion gradients in the microbial cell, depleting the ATP supply, and inhibiting cellular growth (Russell, 1987).

Bacitracin (B), a less commonly fed antibiotic, is a cyclic peptide that can also be fed as a growth promoter (Butaye, et al., 2003) and produces similar effects as M (Russell and Strobel, 1988). It also acts on Gram (+) bacteria (Russell and Strobel, 1988), but unlike M, which disrupts the cell membrane, B inhibits cell membrane synthesis (Siewert and Strominger, 1967). Bacitracin has fermentation effects similar to M, but its effects are during growth of the cell (Russell and Strobel, 1988).

Although M and O have beneficial affects on fermentation when fed alone, *in vivo* studies have shown that when fed together, they do not improve efficiency (Clary, et al., 1993, Zinn and Borques, 1993). Similar results have been found *in vitro*, as Jenkins et al.

(2003) found that when fed together, O and M decreased propionate, increased A:P, and did not change methane (Jenkins, et al., 2003).

Although M likely inhibits Gram (+) bacteria, that description may be an oversimplification. Gram (+) bacteria hydrogenate 18:2 to 18:1 and this step can occur during M supplementation (Fellner, et al., 1997, Sauer, et al., 1998). Lipolytic and hydrogenating bacteria are reported to be mostly Gram (-) and given that M has been shown to inhibit lipolysis, it is hypothesized that M may change the metabolism of Gram (-) bacteria as well as inhibit Gram (+) bacteria (Van Nevel and Demeyer, 1995).

The objectives of the experiment outlined in chapter 4 of this thesis were 1) to determine the effects of antibiotics and oil on rumen fermentation and 2) to monitor the effect of the additives and the sequence of the additives on rumen microbial populations. It was hypothesized that although the changes in fermentation by M, O, and B are similar, they have different effects on the microbial population of the rumen due to their different mode of actions. Because of their different modes of action, the sequence in which the additives are supplemented would have different effects on the populations in the rumen.

Because of limitations with culture-based techniques, molecular methods are necessary to better characterize the populations in the rumen and in other microbial environments. Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu, et al., 1997) has been used to study communities of microorganisms and has been shown to be a specific, reliable, and reproducible method for studying such populations (Dunbar, et al., 2001, Osborn, et al., 2000). Although becoming an established method in microbiology, it is not commonly used to profile populations in the rumen.

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CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

Fat

Fat Supplementation

Animals in high production, such as lactating dairy cattle, are in a negative energy balance because their metabolic requirements are so great. It is very difficult for those animals to consume enough energy to both maintain weight and produce milk. Cows in peak lactation are especially likely to be unable to consume enough energy. To help meet this high demand for energy, both lactating and feedlot cows are often fed increased fat, concentrated grain diets, and ionophores. Either supplemental fat or grain can make the diet more energy dense, but concentrated grain diets put the cow at risk for several disorders such as ketosis, acidosis, and bloat that arise from the rapidly altered ruminal environment. Feeding ionophores such as monensin can lower the incidence of these conditions.

Dairy cattle are fed supplemental fat while in lactation, as fat is very energy dense and does not require reducing the fiber content in the diet. This makes feeding increased fat more energetically efficient than increasing other dietary components. Including 4-7% fat in the diet increases milk production 2-10% more than including just 1-3% fat (Palmquist and Jenkins, 1980). Feeding fat in a protected form can be more energy efficient, as reported in a study by Andrew and colleagues (1991) in which feeding protected fat by forming calcium salts of fatty acids led to a 13.2% increase in metabolic energy (ME) use over diets fed with unprotected fat.

Antimicrobial activity

Although fat can be very beneficial if fed at appropriate amounts, including more than 5% supplemental fat in the diet can be detrimental to ruminal microorganisms (Chalupa, et al., 1986). Too much fat in the diet can decrease milk production and milk fat yield because the high amount of lipids can be antimicrobial if they overwhelm the capabilities of the microbes and inhibit their metabolism (Chalupa, et al., 1986, Van Nevel and Demeyer, 1995). Fat imparts its detrimental effects by coating the feedstuffs and physically blocking access for digestion or by binding to the cell membrane surface of the microbes and thus impeding their function and enzyme production (Palmquist and Jenkins, 1980, Van Nevel and Demeyer, 1995).

Unsaturated fats are more toxic than saturated fats to microorganisms in the rumen, as they are more soluble in the ruminal environment. This makes them available to adhere to both microbes and feed particles, which can inhibit microbial activity or prevent microbial access to the feedstuffs (Chalupa, et al., 1986). Free oil is also detrimental to microbial fermentation (Jenkins, 1993) and is not normally included in the ruminant diet. Oils in unprocessed seeds, however, do not seem inhibitory to microbial fermentation (Dhiman, et al., 1999).

Lipid Metabolism in the Rumen

A drawback of feeding fat to dairy cattle is its fate in the rumen and subsequent effects on milk composition. Numerous factors affect the fate of unsaturated fatty acids in the rumen, and the most extensively studied are those that affect microbial activity or biohydrogenation (Jenkins, 1993). Fatty acids initially enter the ruminal environment in plant lipids either in the esterified or unesterified form (Moore, et al., 1969, Noble, et al.,

1974). Moore and colleagues (1969) studied the fates of both esterified and free fatty acids by infusing the rumen of sheep with either maize oil, which contains esterified linoleic acid (C18:2), or free linoleic acid (for backbone structures of fatty acids, see Table 1). The esterified linoleic acid seemed to allow efficient and rapid biohydrogenation of C18:2 to steric acid (C18:0); there was an initial increase and then a plateau in concentration of oleic acid (C18:1) and the C18:0 concentration increased exponentially. In contrast, the free linoleic acid inhibited the final step of biohydrogenation, as 18:1 was the main product and there was only a small amount of 18:2 that was fully hydrogenated to C18:0 (Moore, et al., 1969).

Nobel and coworkers (1974) also studied the fate of linoleic acid in both esterified and unesterified form in the rumen of sheep, and their results were consistent with those of Moore and colleagues (Noble, et al., 1974). Linoleic acid infused in the triglyceride, or esterified, form was biohydrogenated to a greater extent than when infused as free linoleic acid. The free C18:2 yielded more 18:1 intermediates and fewer C18:0 products when compared to the esterified C18:2. The authors discussed the possibility of there being different pathways for hydrogenating esterified and free fatty acids, as they referred to the possibility of two different microbial groups involved in biohydrogenation: group A to hydrogenate 18:2 to 18:1 and group B to hydrogenate 18:1 to 18:0. They also recognized that the free 18:2 could be inhibitory to the pathway that hydrogenates 18:1 to 18:0. The microbes may be better able to tolerate esterified fatty acids, as the microbes evolved more in their presence than in that of free fatty acids (Noble, et al., 1974).

Lipolysis

Plant lipids, which are present in cattle diets, undergo extensive hydrolysis in the rumen by microbial lipases and hydrolases. This process is called lipolysis, or hydrolysis, and must occur to free the unsaturated fatty acids by hydrolyzing the ester linkages of triglycerides, phospholipids, and glycolipids. Although it happens very quickly, lipolysis may be the rate-limiting step in the formation of saturated fats from unsaturated ones. Microbial lipase carries out this process and yields a fatty acid with a free terminal carboxyl group. This functional group is imperative, as biohydrogenation can occur only on a free fatty acid (Hawke and Silcock, 1969). The free unsaturated fatty acids are then biohydrogenated by the microbial enzymes linoleate isomerase and reductase.

Biohydrogenation

Biohydrogenation occurs after hydrolysis and is a process during which microbial enzymes add hydrogens to unsaturated fatty acids, making them more saturated (Jenkins and McGuire, 2006). Dairy cows most commonly consume the unsaturated fatty acids 18:2, which is found in most seed lipids and concentrates, and linolenic acid (C18:3), which is in forages and thus is consumed during grazing. Linseed and soybean oils also contain high amounts of C18:3 (Palmquist and Jenkins, 1980). The first step in the biohydrogenation of *cis*-9*, cis*-12 C18:2 is the conversion of the *cis*-12 double bond to a *trans*-11 isomer by an isomerase. Next, reductase hydrogenates *cis*-9. Incomplete biohydrogenation can yield *cis*-9*, trans*-11 C18:2 or *trans*-11 C18:1, and complete biohydrogenation yields C18:0.

Bickerstaffe et al. (1972) used goats to study biohydrogenation and found that the most commonly formed fatty acids are *trans*-11 octadecenoic acids and stearic acid. The study also showed that 90% of C18:1, C18:2, and C18:3 fatty acids were biohydrogenated in the rumen of lactating goats (Bickerstaffe, et al., 1972). In a study in dairy cows by Bauchart et al. (1987), C18:3 biohydrogenation was over 92.5% and C18:2 biohydrogenation was between 71.7 and 82.3%. This high 18:3 biohydrogenation may indicate a lack of microbial adaptation to the fatty acid or ruminal microbes could have developed the ability to biohydrogenate unsaturated fatty acids as a mode of protection. In the same study, low amounts of C18:2 were protected in the rumen, perhaps because it was taken up and protected by microbes, but increasing dietary amounts of C18:2 were not protected because the ability of the microbes to take up C18:2 was overwhelmed (Bauchart, et al., 1987).

An *in vitro* study by Fellner (1995) used artificial fermentors to study the ability of the system to predict *in vivo* fermentation. The experiment investigated linoleic acid biohydrogenation, and yielded similar results to *in vivo* studies.

Competition with Methanogenesis

Because biohydrogenation uses hydrogens, it may initially seem to be a way to dispose of excess H's, but that process accounts for less than 2% of the hydrogen pool. Methanogenesis is a major metabolic pathway that serves to dispose of hydrogens. Because both processes use hydrogen atoms, biohydrogenation competes with methanogenesis. Biohydrogenation uses hydrogens before methanogenic archaea do, so not as much methane is produced as waste when hydrogens are being used to hydrogenate fatty acids. This reduction of methane production is beneficial in several

ways. First, the energetically preferred pathway to use hydrogen is biohydrogenation because stearic acid can be used as a source of energy and is not discarded as waste like methane. Also, the interference of biohydrogenation with methanogenesis only influences hydrogen availability; it does not inhibit methanogenic archaea and thus does not interfere with their mode of action (Chen and Wolin, 1979). Finally, reductions in methane production decrease gaseous waste and therefore increase feed efficiency.

Altering Milk Composition

Not only has supplemental fat been fed and studied for decades, but the type, encapsulation, and amount of fat fed to cows also has been extensively researched (Jenkins and McGuire, 2006). Specifically, possible mechanisms to protect lipids from biohydrogenation have been investigated since the 1970's and altering milk composition by nutritional means has been researched since the 1980's (Lawson, et al., 2001). Dairy fat has developed a negative connotation in recent years because of public concern about saturated fat and cholesterol intake, so much research has focused on altering the fatty acid profile of milk. Fat research began with studying how to meet the energy requirements of lactating cows, and the data from those studies as well as more recent ones has helped research on improving milk fat composition (Jenkins and McGuire, 2006). Research on ruminant lipid metabolism began with studying the pathways of lipolysis and biohydrogenation and has evolved into studying the control and manipulation of those processes. Recent research is primarily aimed at improving interactions between fatty acids and microbes to reduce antimicrobial effects while controlling microbial biohydrogenation and thus producing healthier milk fat (Jenkins, 1993). Accordingly, the amount of information about fat in general and the effects of fat

on animal performance also has increased (Jenkins and McGuire, 2006). One important consideration when formulating diets for high producing cows is that milk fat synthesis can drop dramatically with the feeding of high concentrate diets or certain fats (Sutton, 1989). Griinari and colleagues (1998) showed that the amount of fiber and type of fat cause dramatic effects on the milk fat percentage produced, with the combination of low fiber and unsaturated fat showing the greatest amount of milk fat depression (Griinari, et al., 1998).

LC-PUFA

Human Health Interest

For years, the Dietary Guidelines for Americans have urged people to reduce their intake of saturated fat and cholesterol (http://www.health.gov/DietaryGuidelines/). Much research has been aimed at improving the fatty acid profile of milk so that the proportion of saturated fat is decreased and that of unsaturated fatty acids is increased (Jenkins and McGuire, 2006). Meanwhile, consumer awareness of the benefits of unsaturated fatty acids, such as long chain polyunsaturated fatty acids (LC-PUFA), particularly omega-3 LC-PUFA, PUFA, and conjugated linoleic acid (Clary, et al., 1993), is increasing. In particular, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) have been shown to enhance infant growth and visual and cognitive development and reduce the risk of cardiovascular disease, autoimmune disorders, type 2 diabetes, hypertension, rheumatoid arthritis, and certain cancers (Connor, 2000, Simopoulos, 1999).

In spite of these health benefits, intake of these fatty acids from typical diets is usually low. The recommended daily intake of omega-3 PUFA is 650 mg/d, but daily

consumption is less than that in most developed nations. Not only is the n-3 intake in industrialized nations low, but the n-6 intake is high and this raises the n-6 to n-3 ratio to about 20-30:1, up from the healthier 1-4:1 (Simopoulos, 1999). The skewed n-6 to n-3 ratio perpetuates metabolic disorders by increasing pro-inflammatory products, therefore promoting thrombi and atheromas formation, leading to allergic and inflammatory disorders, and contributing to cell proliferation. Increased n-3, on the other hand, produces hypolipidemic, antithrombotic, and anti-inflammatory effects (Simopoulos, 1999). Because the consumption of vegetable oil, which is high in n-6, is increasing, it is important to consume enough n-3 to keep the n-6:n-3 low (Connor, 2000, Simopoulos, 1999, Wijendran and Hayes, 2004). A major natural source of n-3 PUFA is marine oil derived from fish or algae.

Cardiovascular related deaths and illnesses are major health issues in developed nations (Anderson, 2002), making it more important to increase the general public's access to n-3 PUFA. A randomized, controlled trial by Calò and colleagues (2005) investigated the possible use of PUFA on coronary artery bypass graft (CABG) surgery patients for preventing atrial fibrillation (AF) and shortening the postoperative hospitalization. The authors found that the experimental group had significantly reduced incidences of AF and reduced lengths of hospital stays. This was the first study showing PUFA supplementation preventing AF, and the authors hypothesize that these results are due to the anti-inflammatory effects of PUFA. Another benefit to PUFA supplementation is its safety, especially when compared to pharmacological treatment, making PUFA accessible to all patients (Calo, et al., 2005).

Polyunsaturated fatty acids reduce cardiovascular problems in adults and are also essential for pregnant or nursing women and children for ensuring proper growth and optimal brain development. The fatty acids are transported to the fetus during pregnancy and are found in human milk. They are important for proper fetal and infant development (Simopoulos, 1999). In 1990, Ruyle and colleagues investigated the transport of essential fatty acids to the fetus, concentrating on the plasma and erythrocytes. Previous studies had investigated the plasma fatty acid concentrations in both mother and baby, but this was the first to study the role of erythrocytes in fatty acid transport. By sampling blood from mothers and newborn babies, the investigators found that erythrocytes are very important transporters of DHA from the mother to the fetus and transport more DHA to the fetus than plasma. Also, when compared to the fetal transfer of other n-3 fatty acids, DHA transfer is the greatest. The levels of n-3 FA were higher in fetal erythrocytes and in fetal plasma (Ruyle, et al., 1990).

DHA is important for infant development after birth and is supplied by the mother's breast milk. However, infants begin consuming food with low DHA at weaning and may require DHA supplemented baby food. Hoffman and colleagues (2004) studied the effects of DHA supplemented baby food fed daily to weaning infants. One group served as the control and did not receive supplementation, while the other consumed the DHA from 6 to 12 months of age. Some infants in both groups continued to breast feed for about three months of the six-month study. The investigators measured the visual-evoked potential (VEP) acuity and the stereoacuity of the infants at 6, 9, and 12 months of age. Visual-evoked potential was used as a way to compare the retina and visual cortex development between treatments and to characterize visual development as a

whole. The results showed a direct relationship between the blood lipid levels of DHA and visual acuity, and the infants fed the DHA supplemented food had higher VEP acuity at 9 and 12 months, correlating to about 1.5 more lines on an eye chart. In addition, stereoacuity was measured and no significant differences were found. The authors reasoned, based on this and previous studies, that the peak stereoacuity occurs before 6 months of age, and the human milk the infants received during this time was enough for optimal stereoacuity development. This study supports DHA supplementation in the diet of infants until at least one year of age (Hoffman, et al., 2004).

Besides egg yolk, chicken, and oily fish, human milk and supplemented food are the only foods in the typical US diet that are a good source of DHA. As of 2004, no infant formula or baby foods in the United States contained fish (Hoffman, et al., 2004). Mothers are discouraged from eating too much fish because of the risk of metal contamination and, like children, may consume very little or no fish (Oken, et al., 2003). This makes it imperative to provide sources of n-3 PUFA apart from fish or fish oil supplements.

Many groups also have investigated the role of dairy and calcium in preventing obesity in both adults and children (Marques-Vidal, et al., 2005, Mirmiran, et al., 2004, Zemel, et al., 2000), and dairy intake has been the subject of many reviews (Heaney, et al., 2002, Skinner, et al., 2003, Teegarden, 2003). Because of the already beneficial effects of consuming dairy for mothers and children, enrichment of milk with healthful fatty acids would help provide a portion of the recommended daily LC-PUFA allowance.

Incorporation of DHA and EPA into milk fat

One way for the public to increase intake of PUFA is with supplements, but a more practical and economical way to add more PUFA to the diet would be to enrich it in functional foods like milk and milk products. The term functional food describes foods that have benefits other than those listed in the nutrient contents, such as ability to reduce disease risk (Milner, 1999). Milner discussed functional foods and the increased demand the public has for them. He stressed the beneficial role these foods can have, provided the diet as a whole is healthful and the functional foods are not viewed as a "magic bullet." Indeed, the health benefits of the foods are highest when consumed as part of an overall nutritious diet (Milner, 1999).

Attempts to increase the concentration of DHA and EPA in milk fat by adding fish oil to the animals' diet have resulted in low transfer efficiencies of these FA from the diet to milk. The low transfer is likely due to the lack of protection of the oil from biohydrogenation in the rumen. Extensive biohydrogenation of unsaturated fatty acids in the rumen is implicated as a major process that determines the profile of fatty acids supplied to the mammary gland. However, data on the nature and extent of biohydrogenation of EPA and DHA in the rumen is limited and conflicting at best. The fate of AA in the rumen is not known. Thus, strategies to enhance milk fat EPA, DHA, and AA would involve increasing rumen outflow of these fatty acids and their subsequent incorporation into milk fat. The fatty acids may have to be delivered to the rumen in a protected form, such as the raw plant material, rather than the extracted oil.

Numerous groups have attempted to use fish oil to increase LC-PUFA in the milk (AbuGhazaleh, et al., 2002, Donovan, et al., 2000, Lock and Bauman, 2004, Palmquist

and Griinari, 2006). Several studies have incubated rumen fluid with fish oil or pure LC-PUFA to investigate their fate in the rumen, including their possible effects on and involvement in biohydrogenation (AbuGhazaleh and Jenkins, 2004a, AbuGhazaleh and Jenkins, 2004b, Dohme, et al., 2003, Gulati, et al., 1999). These experiments have shown that both the oil concentration in the fluid and the fatty acid concentration in the oil influence fatty aid metabolism (Dohme, et al., 2003, Gulati, et al., 1999).

Gulati et al. (1999) used a range of oil concentrations as they incubated fish oil, squid oil, and max-EPA® at 1-5 mg/ml in sheep rumen fluid. They observed the greatest biohydrogenation at 1mg/ml and the least at 5mg/ml. The fatty acid concentration of the oil used was between 6 and 17% EPA and DHA. The authors noted that C18:2 biohydrogenation occurred and resulted in 18:1-*trans* and 18:0 formation even though EPA and DHA were only hydrogenated to a small extent. The authors noted that when the 5mg/ml oil concentration in the fluid contained different ratios of fish oil and cottonseed oil, the changing ratios caused changes in biohydrogenation, increasing the *trans*-11 18:1:18:0 ratio as the fish oil:cottonseed oil ratio increased (Gulati, et al., 1999).

Dohme and coworkers also used batch cultures to study the effect of fish oil on ruminal fatty acid metabolism, specifically monitoring the effects of oil concentration and fatty acid concentration on lipolysis at 24 and 48 hours. At 24 hours, the increasing concentrations of fish oil had an inverse relationship with lipolysis. At 48 hours, however, the increasing concentration of fish oil initially increased lipolysis and then it occurred at a steady rate. Lipolysis did not differ for EPA and DHA (Dohme, et al., 2003).

Increasing concentration of the oils with the higher fatty acid percentage both increased the concentration of the unesterified fatty acids and decreased their biohydrogenation (Dohme, et al., 2003). Additionally, increased time (48 vs 24 hours) increased both unesterified fatty acids and biohydrogenation (Dohme, et al., 2003). However, because the flasks were re-inoculated, the total oil concentration and the fatty acid concentration in the fluid differed two-fold between the 24 hour and the 48 hour sampling points.

Because soy oil hydrolysis proceeded as normal, the concentration of lipase enzymes should not have affected the rate of lipolysis, but the esterified fatty acids could have affected the activity of the lipase enzymes. Overall, lipolysis seemed to depend most on the amount of oil in the fluid, while biohydrogenation depended on the concentration of unesterified fatty acids, as biohydrogenation was lower in the oil with the greater percentage of EPA and DHA (Dohme, et al., 2003).

Studies that have involved feeding soybean and fish oil to cows have shown an increase in biohydrogenation intermediates such as vaccenic acid (VA; *trans*-11 18:1) and CLA (AbuGhazaleh and Jenkins, 2004b). The striking feature about the increase in intermediates is that it was greater with the combination of fish oil and soybean oil than when either were fed alone, even though fish oil contains little 18:2 and 18:3. This suggests that fish oil influences the metabolism of both oils. AbuGazaleh and Jenkins (2004b) investigated which component in fish oil could be causing the build up of VA and CLA. They used batch cultures of rumen fluid and incubated them with DHA for 24 hours (AbuGhazaleh and Jenkins, 2004b). Based on comparisons of the fatty acid profile of the cultures at 0 and 24 hours, the authors concluded that although DHA is a

component of fish oil that affects biohydrogenation, it might not be the only component affecting it. Docosahexaenoic acid caused a decrease in 18:0 and 18:1 formation compared to other treatments (AbuGhazaleh and Jenkins, 2004b).

In another study, the fate of both DHA and EPA in batch cultures of rumen fluid was investigated (AbuGhazaleh and Jenkins, 2004a). Pure forms of DHA and EPA were used to study the biohydrogenation by rumen microbes. Different levels of DHA (0, 5, 10, 15, and 20 mg) and EPA (0, 5, 10, and 15 mg) were tested to determine the effect of level of fatty acid on biohydrogenation of DHA and EPA. The amount of DHA and EPA in the cultures decreased over time, with the disappearance and the concentration of the added fatty acids exhibiting an inverse relationship. At similar concentrations, a greater proportion of EPA disappeared compared to DHA. It has been suggested that DHA and EPA could have been biohydrogenated, their carbon chain could have been shortened, or they could have been transformed into other C22:6 and C20:5 isomers, respectively (AbuGhazaleh and Jenkins, 2004a). Increases in unsaturated fatty acids with addition of DHA and EPA raise the possibility that DHA and EPA could have influenced the unsaturated fatty acid concentration whether directly, via shortening of their carbon chain, or indirectly, by inhibiting the activity of the enzyme reductase of ruminal microorganisms (AbuGhazaleh and Jenkins, 2004a).

In 1999, Franklin and colleagues conducted the first study to investigate the effects of feeding the marine algae *Schizochytrium* sp. to dairy cows. Previous studies focused primarily on feeding fish oil to cows and other ruminants, but this was the first study in which they fed algae to dairy cows. The algae contained DHA as 5.52% of the total fat and yielded 0.46% of the milk fat as DHA, with the control having 0% of the

milk fat as DHA. Not only did feeding algae show increased amounts of unsaturated fatty acids in the milk, but also it showed decreased levels of saturated fatty acids (Franklin, et al., 1999).

CLA

Endogenous Synthesis

Partial biohydrogenation of linoleic acid may lead to two conjugated double bonds in the fatty acid, which make up isomers called conjugated linoleic acid (Clary, et al., 1993). The most common positions for these double bonds are the 9^{th} and 11^{th} carbons or the 10^{th} and 12^{th} carbons counting from the carboxylic end. Hydrogenation can create bonds in either the *cis* or the *trans* position (Lawson, et al., 2001).

CLA isomers are naturally found in milk; biohydrogenation of linoleic acid (LA) in the rumen is one source of CLA production. Biohydrogenation of C18:2 yields many different isomers as intermediates, of which *cis*-9, *trans*-11 is the most common (Palmquist, et al., 2004) and also is found in milk fat (Parodi, 1976). It was originally thought that production of CLA as an intermediate in ruminal biohydrogenation accounted for its presence in milk. However, not only can the CLA produced in the rumen be incorporated into milk, but the *trans*-11 18:1 formed in the rumen can serve as a substrate for endogenous synthesis, as about a third of the fatty acid is taken up by the mammary gland, where it can be desaturated (Corl, et al., 2001, Griinari, et al., 2000). The site of endogenous synthesis varies with the stage of production of ruminants. By studying delta-9 desaturase activity, Corl et al. (2001) determined that adipose tissue and the mammary gland are the primary sites of endogenous CLA synthesis in growing and lactating ruminants, respectively.

Dairy cows abomasally infused with linoleic acid yielded at least 31% more CLA in their milk than controls, providing strong evidence for endogenous CLA synthesis (Griinari, et al., 2000). Another experiment used both a stimulator and an inhibitor of delta-9 desaturase and those results showed that the enzyme uses *trans*-11 C18:1 as a substrate to synthesize CLA in cows. The authors estimated that endogenous synthesis of *cis*-9, *trans*-11 accounted for more than half of its presence in milk fat (Griinari, et al., 2000). Subsequent studies have projected that endogenously synthesized *cis*-9, *trans*-11 makes up as much as 80-97% of the isomer found in milk fat (Lock and Garnsworthy, 2002, Palmquist, et al., 2004).

Health Benefits

In the early 1930's, scientists unwittingly began the study of CLA, as they recorded changes in the fatty acid profile of milk while studying possible fluctuations in the vitamin concentration of milk due to changes in the season (Booth, et al., 1935). In addition, still before the characterization of CLA, another group showed changes in fatty acid adsorption due to dietary changes. Eventually, Parodi (1976) used gas liquid chromatography (GLC) to show that the major conjugated milk fatty acid is *cis*-9, *trans*-11 octadecadienoic acid, and later proposed the name rumenic acid (RA) for the isomer (Kramer, et al., 1998).

After identification of RA, Pariza and coworkers found grilled ground beef to have antimutagenic activity that varied with time and temperature (Pariza, et al., 1979). They also reported CLA to have anticarcinogenic activity (Pariza and Hargraves, 1985). Thus began a flood of research on this fatty acid found in milk that has since been shown

to have a wide array of biological effects, including anticarcinogenic activity, reductions in atherosclerosis, better immune system function, and improved body composition.

A 1997 review by Parodi summarized the current studies on CLA and its potential role as an anticarcinogen, emphasizing the importance of preventative measures to combat cancer. CLA had significant effects on inhibiting human malignant cell lines of melanoma, colorectal, breast, and lung cancer and caused marked differences in precursors for mouse and rat cancers (Parodi, 1997).

Hundreds of animal cancer trials have shown CLA to have anticarcinogenic properties. Ip and Pariza began these, and Ip has conducted numerous cancer trials using CLA, concentrating on mammary cancer. One such study fed CLA enriched butter and commercial CLA to rats (Bauman, et al., 2000, Ip, et al., 1999). This was the first *in vivo* study to feed CLA made mostly of the *cis*-9 *trans*-11 isomer in a food as well as a mixture of CLA isomers in free fatty acid form. Both the rats fed the butter and those fed the fatty acid preparation showed reduced risk of mammary cancer. This is an important finding, as research on CLA in animal models had been carried out with a mixture of isomers (Ip, et al., 1999).

Lipid metabolism and body composition changes occur upon *cis*-10*, trans*-12 supplementation and not with *cis*-9*, trans*-11, so it is likely that the other CLA isomers may also play a role in the health benefits caused by mixtures of CLA, although made predominantly of the *cis*-9*, trans*-11 isomer. *Trans*-10*, cis*-12 CLA has been shown to cause milk fat depression in dairy cows (Griinari, et al., 2000, Griinari, et al., 1998) and weight loss in an obese line of mice (House, et al., 2005), so it is possible that there are other biologically active CLA isomers (Cook and Pariza, 1998; Lock and Bauman, 2004).

Cook and Pariza began investigating the role of CLA in the immune response, as a challenged immune system often results in weight loss and a dietary supplement would be a useful way to combat wasting. Animals fed CLA have maintained both their feed intake and body weight when immune challenged, rather than exhibiting the wasting effects that the control animals, which were fed either linoleic acid or fish oil, experienced. CLA was able to maintain the weight of the animal without compromising the function of the immune system. Studies using chickens as a model and subsequent ones with rats and mice showed that CLA-fed animals had less anorexia and wasting due to a challenged immune system and the control diets offered no protection to the animals (Cook, 1999, Cook, et al., 1993). These studies demonstrate the potential to use CLA, rather than an immunosuppressant, to control body weight during an immune response.

Incorporation into milk fat

Over half of the CLA formed in a lactating dairy cow is synthesized endogenously from *trans*-11 C18:1, rather than as an intermediate in biohydrogenation (Griinari, et al., 2000). It is likely, therefore, that the most efficient way to increase the CLA in milk is to focus on dietary formulations that promote *trans*-11 C18:1 production in the rumen (Griinari, et al., 2000). Endogenous synthesis and biohydrogenation can be influenced by the diet of the cow. *Cis*-9, *trans*-11 CLA is the most abundant CLA isomer in the rumen and in milk. Strategies to increase *cis*-9, *trans*-11 in milk include increasing both the isomer and *trans*-11 18:1 in the rumen. The only known way to increase *trans*-10*, cis*-12 in the milk is to increase the isomer in the rumen, as it is not endogenously synthesized (Lock and Bauman, 2004). The concentration of *trans*-10*, cis*-12 CLA in the

rumen increases with diets that are implicated in milk fat depression (Lock and Bauman, 2004).

Biohydrogenation is the major obstacle to increasing the levels of both PUFA and CLA in milk. Feeding fat in a protected form to the ruminant is necessary to prevent it from being biohydrogenated. Cows fed protected algae produced milk with more than seven times as much CLA than was found in the milk produced by the cows fed the control diet (Franklin, et al., 1999). This could be because of the effects of LC-PUFA on biohydrogenation and the possible accumulation of intermediates, particularly *trans*-11 18:1, which can be desaturated in the mammary gland to *cis*-9, *trans*-11 CLA (Corl, et al., 2001). Several other studies that have fed fish or other marine oils have also shown increased CLA in the milk fat from the cows (Chouinard, et al., 2001, Whitlock, et al., 2002). Several variables affect CLA concentration in milk fat, but the most significant are changes in diet and differences between the animals themselves, such as desaturase activity (Palmquist, et al., 2004).

Antibiotics

History of Feeding

Antibiotics have been used in animal feed at subtherapeutic levels for decades. Debates occurred in the 1960's over possible antibiotic resistance of bacteria that affect both humans and animals, especially gram-negative bacteria such as *E. coli* and *Salmonella* spp. The discussions led to limited FDA approval of antibiotics, allowing only ionophore antibiotics, in the US (Russell and Strobel, 1989). Monensin has been fed to beef cattle in the United States since 1975 and is the most commonly tested and used ruminant ionophore to date (for structure, see Figure 2). In general, when antibiotics are

fed to ruminants, they alter the microbial population within the rumen to exert influence on fermentation products (Pressman, 1976). Both carboxylic ionophore antibiotics and cyclic peptide antibiotics have been used as feed additives and have similar effects on animal production (Butaye, et al., 2003). Ionophores are classified as antibiotics because of their toxicity to many bacteria, protozoa, fungi, and other organisms (Pressman, 1976). They are lipophilic substances with hydrophobic exteriors and hydrophilic interiors. Cell membranes are made of lipid bilayers, so the hydrophobic exterior of the ionophore is able to pass through the membrane of the bacteria, while its hydrophilic interior can bind to ions and transport them into and out of the cells by shielding and delocalizing the ionic charge. Ionophores can be uniporters or antiporters; uniporters facilitate unidirectional transport of a single ion, while antiporters can bind to and transport more than one ion (Butaye, et al., 2003).

Monensin

Monensin is an antiporter that is produced by *Streptomyes cinnamonensis* (Butaye, et al., 2003). It is the most commonly fed ionophore antibiotic, and has affinity to Na+ and to a lesser extent, K+ (Russell and Strobel, 1989). Although monensin's affinity for Na+ is about 10 times greater than its affinity for K+, the concentration gradient across the cell membrane of K+ is 25 times greater than that of Na+, making it energetically favorable for monensin to transport K+ out of the cell rather than transporting Na+ into it. As monensin transports K+ out of the cell, it transports H+ in, leading to an increased H+ concentration in the cell. ATP is used to pump out the H+, which depletes the ATP supply. The reduction in ATP is likely a major cause of cellular growth inhibition after monensin treatment (Russell, 1987).

Monensin is most active against Gram (+) bacteria, which lack much of the protective outer membrane that makes Gram (-) bacteria less susceptible to the antibiotic (Russell, 1987). Monensin is 670 Daltons, and molecules greater than 600 cannot penetrate the cell walls of many Gram (-) bacteria, aiding to their natural resistance (Butaye, et al., 2003, Russell and Strobel, 1989). Peptidoglycan surrounds the bacterial cell membranes of both Gram (+) and Gram (-) bacteria. However, it is much thicker and makes up 40-90% of the dry weight of the cell wall of Gram (+) bacteria, but is less than 10% of the weight of the cell wall of Gram (-) bacteria (Stanier, et al., 1976). Chow et al. (1994) used radiolabeled monensin and showed that the lipopolysaccharide cation bridges on the outer membranes of ruminal bacteria are necessary for binding monensin. They also showed that resistance to monensin is likely more complex than cell membrane characteristics (Chow, et al., 1994).

Monensin's affects on cattle include decreasing feed intake by 4% while increasing milk yield and either maintaining or improving average daily gain, and also reducing methane production (Duffield, et al., 2002, Fellner, et al., 1997, Goodrich, et al., 1984, Rumsey, 1984, Tedeschi, et al., 2003, Thornton and Owens, 1981). It has been suggested that the effect on methane reduction is attributed to the inhibition of hydrogen producing bacteria, which decreases the substrate needed for the growth of methanogens (Chen and Wolin, 1979). Monensin also selects for succinate producing bacteria or for those that produce propionate from succinate, resulting in an increase in propionate concentration. (Russell and Houlihan, 2003). Between 2 and 12% of GE intake is lost as methane (Johnson and Johnson, 1995), and by lowering methane and capturing the carbon as propionate, monensin can lower the energy losses by 30% (Schelling, 1984).

As discussed, methanogenesis is the primary pathway that disposes hydrogen produced during microbial fermentation. This can occur with carbon dioxide or a methyl group as a substrate that gets reduced. Carbohydrate-fermenting bacteria can produce hydrogen and carbon dioxide which methanogenic archaea can use to form methane (Hungate, 1967). Because methanogens are Archaea, they differ from Bacteria in many ways, including the absence of the peptidoglycan polymer of the cell wall (Fox, et al., 1977, Wolin and Miller, 1987). Therefore, antibiotics that target the peptidoglycan component of the cell wall do not inhibit methanogens (Van Nevel and Demeyer, 1977). Methanogens can use formate, acetate, methanol, and mono-, di-, and tri-methylamine to form methane, but most rumen methanogens use hydrogen and carbon dioxide to produce methane (Wolin and Miller, 1987). In the ruminant, acetate has not been shown to be a major substrate for methanogenesis (Hobson and Stewart, 1997).

Health Effects of Monensin

Monensin has beneficial effects on feed efficiency and milk production, and has been shown to reduce the occurrence of many common ailments in cattle. It can prevent acidosis and bloat, in part due to its negative effects on *S. bovis*, a Gram (+) lactic acid producing bacteria (Russell, 1987), reduce coccidiosis in calves (Fitzgerald and Mansfield, 1973), and lower tryptophan-induced acute bovine pulmonary edema and emphysema, a respiratory disease of adult cattle (Hammond, et al., 1978). Also, it decreases the incidence of ketosis (Sauer, et al., 1989), displaced abomasums, and retained placentas (Duffield, 2000).

Monensin is not used in human or veterinary medicine because of its demonstrated oral toxicity. Great care must be taken to accurately calculate feed

formulations, such as avoiding transposing a decimal place, as doses ten times the recommended daily intake have caused anorexia and even toxicity in dairy cows (Gonzalez, et al., 2005, Potter, et al., 1984).

Bacitracin

Bacitracin, produced by *Bacillus licheniformis* strains (Siewert and Strominger, 1967), is a cyclic peptide antibiotic that can also be fed as a growth promoter (Butaye, et al., 2003) and produces similar effects as monensin. It also acts on Gram (+) bacteria (Russell and Strobel, 1988), but unlike monensin, which disrupts the cell membrane, bacitracin inhibits cell membrane synthesis (Siewert and Strominger, 1967). Because both monensin, which is an ionophore, and bacitracin, which is a polypeptide, act on Gram (+) bacteria, they can be used in comparative studies to elucidate pathways and better understand their mode of action. One such study illustrated the similar effects the two antibiotics have on methane production and other parameters (Russell and Strobel, 1988).

Peptidoglycan is an integral component of the cell membrane of bacteria, and bacitracin inhibits its synthesis by preventing dephosphorylation of a lipid carrier in the peptidoglycan synthesis pathway. In a study by Stiewert and Strominger (1967), bacitracin was the only antibiotic tested that inhibited both bacterial cell wall synthesis and the dephosphorylation of lipid pyrophosphate. By preventing this step, bacitracin prevents the lipid pyrophosphate from recycling into the cell wall synthesis cycle (Siewert and Strominger, 1967).

Particular genes may be involved in natural resistance to bacitracin, such as *bacA*, which may allow for recycling of the carrier C55-isoprenyl phosphate by phosphorylating

undecaprenol. Because *bacA* homologues are found in bacitracin susceptible and bacitracin resistant bacteria, its regulation, and not just its presence, likely plays a role in the microbial response to bacitracin (Chalker, et al., 2000).

Antibiotic Resistance

Antibiotics have been used since the 1940's, and for years it seemed that humans were going to overcome microbial infections and diseases. However, microbial resistance to antibiotics was observed shortly after their introduction and created a greater awareness of the properties of bacterial populations and the possible consequences of non-judicious use of antibiotics. Despite early awareness of bacterial resistance, doctors today are likely to prescribe antibiotics because the prescription will appease the client even when antibiotics will have no affect on the patient's ailment, such as a viral infection (Vogt and Jackson, 2001). When patients take an antibiotic that is not directly treating a disease, it is being administrated at a subtherapeutic level (Vogt and Jackson, 2001). This level has adverse affects on the bacteria naturally occurring in the body and disrupts the balance of microorganisms, which could allow resistant and possibly harmful bacteria to cause illness in the patient. Therefore, misuse of antibiotics has the potential to actually create health problems. Exacerbating the problem of over prescription is misusing the antibiotics. Patients very often stop taking their prescribed antibiotics when they begin to feel better, or they forget and miss doses. Terminating treatment early provides an opportunity for resistant bacteria to flourish, because the exposure to the antibiotic has killed or inhibited susceptible organisms and thus reduced the competition facing resistant bacteria (Vogt and Jackson, 2001).

Although the practice of feeding antibodies to production animals has benefited the animal industry, it has also raised concerns in the general public about possible antibiotic resistance and the impact on consumers. While human overuse and misuse of antibiotics is alarming, the most heated debate over antibiotic use involves their administration to production animals (Vogt and Jackson, 2001). Giving antibiotics to livestock at subtherapeutic levels may cause the same problems that develop from human misuse of the drugs. Major concerns about the practice include humans' unwittingly consuming food contaminated with resistant bacteria and animals excreting antibiotics in an intact form. This could allow antibiotics to not only potentially select for resistant bacteria in both the soil and water, but also for both antibiotics and resistant bacteria to be in the human water supply (Vogt and Jackson, 2001).

Literally tons of antibiotics are used in agriculture, and there is conflicting data on exactly how much is used as growth promoters or prophylacticly at subtherapeutic levels or to treat disease at therapeutic levels (Salyers and Whitt, 2005). Public and scientific concern stems from possible creation and promotion of resistant strains with feeding antibiotics. It would be possible for these strains to contaminate the animal carcass and enter the human intestinal tract, where they may transfer resistant genes (DNA) to the microorganisms that normally inhabit the human colon through a process known as conjugation. Horizontal gene transfer (HGT), primarily through conjugation, is an important factor in determining the likelihood of developing antibiotic resistance. A concern with feeding antibiotics to dairy cattle would be their appearance in the milk. Pasteurization should kill the bacteria, but consuming milk with antibiotics intact in it could be potentially dangerous (Salyers and Whitt, 2005).

Mechanisms of Antibiotic Resistance

Bacteria may become resistant to antibiotics through three known mechanisms. First, they can produce proteins that modify the drug to inhibit its antimicrobial activity. For example, bacteria that have gained resistance to penicillin may produce the β-lactamase enzyme that renders the compound ineffective by cleaving its active region, the β-lactam ring (Salyers and Whitt, 2005). Second, bacteria may combat antibiotics by preventing entry of the majority of the drug, making its concentration inside the bacteria below the effective level. Tetracycline resistant bacteria utilize this method by producing proteins that serve as efflux pumps, which physically pump the antibiotic out of the cell (Salyers and Whitt, 2005). Finally, bacteria can resist antibiotics by mutating to alter the binding region of the drug. Streptomycin resistant bacteria mutate the ribosomal protein to which the antibiotic binds (Salyers and Whitt, 2005).

Resistant bacteria are able to spread through three main routes: transformation, transduction, and conjugation. These routes of passing on mutations do not involve inheritable traits and thus do not require reproduction, and often do not even require interaction with the same species for transfer of resistant genes (Vogt and Jackson, 2001). In fact, the process of transduction involves the transfer of DNA from a bacterial virus, a bacteriophage, to bacteria. Transformation occurs when bacteria take up genes or gene fragments from other cells in its environment. In the laboratory, this process occurs during heat shock and cooling so cells that have been prepared to be competent can take up the DNA. In nature, it may occur when cells lyse and neighboring cells take up their genetic contents. Along with the discovery of bacterial transformation came the first knowledge of bacterial recombination. This finding led to a search for a bacterial event

resembling mating in eukaryotes, which was called conjugation. This process requires cell-to-cell contact and is the transfer of genetic material from a donor cell to a recipient cell. Conjugation is a unique mating process in that it transfers a plasmid and not the bacterial chromosome. A plasmid may possess genes for its own replication, that promote its transfer, or that can confer certain properties to its host such as antibiotic resistance (Stanier, et al., 1976).

Along with the transduction process, conjugation may occur between virtually any species of cells. De la Cruz and Davies (2000) discussed the role of horizontal gene transfer in spreading antibiotic resistance. Similarities in gene transfer across species, such as the ability of bacteriophages and retroviruses to insert their DNA via recombination into prokaryotic and eukaryotic genomes, respectively, highlight the extensive role of HGT (de la Cruz and Davies, 2000). Ferguson and colleagues (2002) studied the possible horizontal gene transfer of human epithelial cells and found that HGT can and does occur between human cells, thereby providing an avenue for antibiotic resistance to spread within an infected individual (Ferguson, et al., 2002). Once resistance is acquired, bacteria can pass antibiotic resistance on to subsequent generations, which would exponentially increase the prevalence of such traits given the rapid proliferation of bacteria (Vogt and Jackson, 2001).

Resistance to Monensin

Multiple studies have investigated monensin resistance in ruminal microorganisms. Dawson and Boling (1983) suggested that monensin increased resistant bacterial populations in treated calves. The difference in the monensin resistant populations in the treatment and control animals were due in part to a decrease of

monensin resistant bacteria in the controls and not just an increase of monensin resistant bacteria in the treatment animals. Had the resistant population not dropped, the difference between populations would have only been about 20%. Because the resistance in the controls did decrease, the difference between groups of calves was 60%. Nonetheless, monensin supplementation did appear to increase the population of bacteria resistant to monensin (Dawson and Boling, 1983). Also, resistant bacteria were present in control animals that had never been exposed to the antibiotic (Dawson and Boling, 1983). Once monensin was withdrawn, susceptible bacteria levels returned close to pretreatment amounts (Dawson and Boling, 1983).

Given monensin's affinity for K+, potassium concentration is used as an indicator of monensin action. Using sodium concentration as an indicator is more difficult because there is so much sodium outside the cell already and it binds to ruminant bacteria with varying levels of specificity (Russell and Strobel, 1989). Lana and Russell (1996) monitored K+ kinetics to study monensin resistance. Cows were fed monensin for 18 days and rumen samples were taken before, during, and after the treatment period. Rumen fluid was taken from the animals to measure potassium depletion from microorganisms. The fluid was centrifuged and the microorganisms were resuspended in potassium containing medium and the potassium concentration of the control and those treated with a glycolytic inhibitor, toluene, monensin, or lasalocid, were measured. All treatments caused a rapid decrease in cellular [K+], and the ionophores decreased the cellular [K+] by 50%, suggesting a natural resistance of the bacteria to monensin (Lana and Russell, 1996). However, the bacteria may have become dormant after monensin treatment, making further K+ depletion by the ionophore improbable. Bacteria from the

cows fed monensin had an increased Kd, or an increased concentration of monensin required to remove half of the intracellular K+ (Lana and Russell, 1996). Once monensin was withdrawn, the Kd of those samples was comparable to pretreatment levels (Lana and Russell, 1996). Measuring K+ concentration *in vitro* is a useful tool because the [K+] of the medium can be closely monitored, but the same measurements could be inaccurate *in vivo* because of the rapidly changing ruminal environment.

Sauer et al. (1998) conducted an extensive study on lactating dairy cows to investigate the effects of feeding monensin to cows that had already been exposed to monensin and withdrawn from the antibiotic. During the first exposure to monensin, the cows exhibited classical responses, including improved feed efficiency, increased milk production and propionate, and decreased CH_4, milk fat, biohydrogenation, and A:P. Control animals were included during the second trial. It was their first exposure to the antibiotic, and they exhibited classical responses to monensin. However, the cows that had been previously exposed to monensin did not exhibit the same responses to the ionophore as the controls. These results do not confirm antibiotic resistance, but they do indicate the possibility of microbial adaptation or population shifting due to prior exposure (Sauer, et al., 1998). These are limited data on long term use of ionophores in production animals (Fellner, et al., 1997).

Monensin and Fat Interactions

Zinn and Borques (1993) fed monensin to steers receiving fat supplemented diets. The ionophore and fat addition had few significant effects on growth performance, carcass characteristics, and digestion parameters when compared to the control steers receiving the fat supplemented diets without monensin. Average daily gain, dry matter

intake, and feed efficiency were not affected. Monensin did increase the percentage of propionate and decreased both butyrate and methane production. The results indicate that feeding both monensin and fat to steers receiving a high-energy diet may not have additive effects on growth performance (Zinn and Borques, 1993).

Steers fed high concentrate diets and either fat, ionophores, or both were evaluated for their growth performance and fermentation end products (Clary, et al., 1993). On cows fed monensin, supplemental fat tended to decrease average daily gain, and on cows fed supplemental fat, monensin decreased both dry matter intake and average daily gain. Alone, fat and monensin had effects on the steers, but when fed together, the characteristics of the steers were similar to control animals (Clary, et al., 1993). Monensin decreased A:P, but when supplemental fat was added, A:P did not differ from the steers fed only supplemental fat or from the steers receiving the other treatments. Therefore, adding both monensin and supplemental fat may not improve the efficiency of the animals on a high-energy diet (Clary, et al., 1993).

Fellner et al. (1997) investigated the effects of ionophores on biohydrogenation and reported reduced saturated fatty acids in rumen cultures in the presence of monensin. Monensin supplemented fermentors produced 11.5% less 18:0 before the addition of 18:2 and 15% less after supplementation with linoleic acid. Not only did the amount of the saturated fatty acids decline, but the rate of C18:0 production also decreased. The reduction in 18:0 concentration and production suggests an inhibitory effect of the ionophores on biohydrogenation. In the presence of monensin, addition of linoleic acid increased *cis*-9, *trans*-11 CLA, while *trans*-10, *cis*-12 CLA and *trans*, *trans*-18:2 proportions decreased. Also, both *cis*- and *trans*-18:1 increased in the fermentors

receiving ionophores, both before and after supplemental fat addition. *In vivo*, monensin decreased total milk fat production, decreased saturated fatty acids in the milk, and increased total 18:1 and the *trans*-18:1 isomer (Sauer, et al., 1998).

Jenkins et al. (2003) researched the interactions between fat and monensin supplementation *in vitro* when administered with a high-energy diet. Fermentors on a high corn or barley diet received monensin or soybean oil and then began receiving both additives. When fed alone, soybean oil and monensin increased propionate, decreased A:P, and decreased methane production. However, when fed together, propionate decreased and A:P and methane did not change (Jenkins, et al., 2003). The combination of the two caused a decrease in *trans*-10 18:1, so adding both monensin and soybean oil to the diet may contribute to milk fat depression.

Other studies reported a decrease in lipolysis with the addition of monensin and fat both *in vitro* and *in vivo* (Van Nevel and Demeyer, 1995). Describing ionophores as inhibiting Gram (+) bacteria may not be the most accurate description, as Gram (+) bacteria hydrogenate 18:2 to 18:1 and this step can occur during monensin supplementation (Fellner, et al., 1997, Sauer, et al., 1998). Lipolytic and hydrogenating bacteria are reported to be mostly Gram (-) and given that monensin has been shown to inhibit lipolysis, it is hypothesized that monensin may change the metabolism of Gram (-) bacteria as well as inhibit Gram (+) bacteria (Van Nevel and Demeyer, 1995).

Bacterial Populations

SAB and LAB

Bacteria can be divided into two groups based on their location in the rumen: those that are present in the solid, or mat, phase of digesta called solid associated bacteria

(SAB) and those that occur in the liquid phase of digesta called liquid associated bacteria (LAB) (Legay-Carmier and Bauchart, 1989). Accordingly, these groups of bacteria have different properties, modes of action, and functions. The enzymes produced by these two populations have different activity levels, as the bacteria in the SAB need enzymes to break down cell walls of plants and the bacteria in the LAB need enzymes to break down soluble saccharides. A third division of bacteria was originally proposed, a mixture of LAB and SAB (LAB+SAB), but the chemical compositions of LAB and LAB+SAB are similar enough to be classified into LAB (Legay-Carmier and Bauchart, 1989).

Once fatty acids are freed from their esterified form by lipolysis, they can be rapidly hydrogenated and become part of the lipid fraction of SAB (Demeyer and Doreau, 1999, Lawson, et al., 2001). Fatty acids bind to SAB at higher concentrations than they do to LAB, which may be due to greater accessibility to carbohydrates on solid feed, especially the finer particles of the feed (Legay-Carmier and Bauchart, 1989).

Group 1, 2, and 3 Bacteria

In 1976, the ruminal bacteria were roughly divided into three groups based on their mode of action and end products of fatty acid metabolism (Hazlewood, et al., 1976). Previously isolated organisms that could hydrolyze phosphatidylcholine were found to hydrogenate PUFA and yielded unique end products, showing that lipolysis and biohydrogenation could occur in the same organism. However, among a separate group of organisms isolated for their biohydrogenation abilities, not all of them were lipolytic. This comparison shows that biohydrogenation may be a specific adaptation instead of a general way to discard extra hydrogens (Hazlewood, et al., 1976). The three groups of end products included: the most lipolytic group that did not yield 18:0, the less lipolytic

group that did not yield 18:0, and the least lipolytic group that did produce 18:0 (Hazlewood, et al., 1976).

Group A and B Bacteria

In 1984, Kemp and Lander differentiated biohydrogenating microbes into Group A and B bacteria. Group A hydrogenates the *cis*-9 bond of linoleic acid to form *trans*-11 octadecenoic acid, while group B bacteria can hydrogenate both *cis* and *trans* octadecenoic acids to stearic acid, a saturated fatty acid. In fact, in their study of six rumen bacteria, group A bacteria did not produce any steric acid. The products of group B microbes can be more complicated because they can produce isomers from octadecenoic acid. Because of their specific roles in different steps in the biohydrogenation pathway, it is important to have a mixed culture of microorganisms for complete biohydrogenation. The hydrogenation step performed by group B bacteria may be rate limiting, a characteristic underscored by the thought that unsaturated fatty acids may be more toxic to group B bacteria than to group A (Palmquist, et al., 2004). The results do not explain the purpose of biohydrogenation, but do support the hypothesis that biohydrogenation may have positive effects for the microbes, which hydrogenate and therefore alter potentially harmful molecules (Kemp and Lander, 1984).

Van de Vossenberg and Joblin (2003) specifically studied the ruminal bacteria *Butyrivibrio hungatei* and showed that it can biohydrogenate both linoleic and linolenic fatty acids. This contradicts having a strict division between group A and group B bacteria, as *B. hungatei* is capable of performing both hydrogenation steps and thus has properties of both groups of bacteria. It also shows that complete biohydrogenation may

not always require bacteria from both group A and B (Palmquist, et al., 2004, van de Vossenberg and Joblin, 2003).

Culture-Based Techniques

Before molecular techniques were developed, studies of microbial communities were limited to culture-based methods. Therefore, all knowledge of the identity of the organisms in the rumen was because of culture work. Culturing microorganisms is an invaluable technique in microbiology, and its importance should not be overlooked. However, when identifying the species present in a community, culture-based techniques have significant drawbacks (Dunbar, et al., 1997).

One of the most important concerns with using culturing to study a population is that not every organism in the population can be cultured. In fact, cultivable microorganisms may make up as little as 0.1-5% of the total microbial population. That leaves an astounding proportion of bacteria that simply cannot be studied or even documented. Therefore, much of what is known about microbial communities through culture methods is a small introduction to the rest of the population, and even after the culture has been established, there are still biases (Dunbar, et al., 1997).

In order to culture an organism, it must be taken from its natural environment so it is no longer exposed to the other organisms, nutrients, and interactions between the organism being cultivated and the environment, which may have helped or hindered its growth, development, and behavior. By simply isolating an organism, investigators introduce many variables. The organism may thrive when it does not face competition from other organisms, or it may suffer from not having access to the metabolic products of its prior neighbors. Also, when experimenters introduce the growth media, it may be

so different from the natural environment that it could alter the microorganism significantly. An additional problem with liquid cultures is that they can select for rapidly growing organisms and inhibit the efficient growth of slow-growing organisms (Dunbar, et al., 1997).

Dunbar and coworkers (1997) investigated biases introduced during culturing by comparing batch culture enrichments and direct plating. Molecular techniques such as PCR, restriction enzyme digestions, and probe analysis were used to identify the 2,4-D-degrading bacteria present in the cultures and on the plates. The investigators showed that direct plating identified many more organisms than the liquid culture method, as it revealed 25 distinct populations while the liquid cultures only revealed 7, but they also noted that direct plating could miss strains. Only the species able to form colonies will be observed with direct plating, while those species might be detected if they are competitive enough in the conditions of the liquid cultures. However, the authors hypothesized that these organisms would have been detected within direct plating had a larger sample size been used. It is difficult to determine the ideal sample size so all organisms will be detected even with direct plating, but most importantly, liquid cultures consistently underestimate species diversity within a microbial population (Dunbar, et al., 1997).

Upon exposure to 2,4-D gene probes, the genomic DNA from the colonies obtained from direct plating revealed three groups of 2,4-D catabolic genes, while 98% of those from the liquid culture showed the same hybridization pattern. This is most likely because the direct plating method separated the competing populations so more could

grow. The liquid culture did not separate the groups, so only one thrived in that environment (Dunbar, et al., 1997).

Non Culture-Based Techniques

Because of limitations with culture-based techniques, molecular methods are necessary to better characterize the populations in the rumen and in other microbial environments. It is possible to isolate the bacterial DNA from cultures and perform PCR to determine the presence of the microbial species, as well as cell counts to determine the number of the microorganisms present. By determining how the microbes in the rumen change upon exposure to different diets, including supplemental fat and antibiotics, more will be known about the pathways leading to possible shifts in populations and thus how to more efficiently manipulate the rumen environment.

Molecular methods to study microorganisms include using probes to hybridize to nucleic acids in unknown samples, restriction fragment length polymorphism (RFLP), clone libraries, rtPCR, and most recently, terminal restriction fragment length polymorphism (T-RFLP). Several groups have begun using these alternatives to culture-based techniques to study the microbial communities in the rumen. 16S rRNA probes have been used to study monensin's effects on the ruminal bacteria (Stahl, et al., 1988) as well as other dietary effects on the microbes (Briesacher, et al., 1992). 16S rDNA libraries and real-time PCR are additional methods that have been used to investigate possible bacterial shifts in response to dietary changes (Tajima, et al., 2001, Tajima, et al., 1999, Tajima, et al., 2000, Whitford, et al., 1998).

16S rRNA

Molecular techniques have complemented culture-based methods for studying populations of microorganisms for decades. Woese first wrote about the three ancestral lines based on phylogeny with Fox in 1977. The three domains were called urkingdoms and the divisions were tentative and speculative (Woese and Fox, 1977). In 1990, Woese and colleagues formally proposed that life be organized into three domains: Archaea, Bacteria, and Eukarya, rather than the accepted five kingdom system or the prokaryote-eukaryote division. The domain classifications were based on molecular systems that all domains shared, but were distinctive enough across domains to necessitate a separation. In particular, the small subunit rRNA is present across all domains, can be isolated, and because it had been so extensively studied, its structure was known for many organisms. Both nucleotide sequence and secondary structure information on rRNA were used to help create the domain classifications (Woese, et al., 1990).

Even when cultured, comparisons among closely related species are difficult because phenotypic characteristics can be ambiguous. Using nucleic acid sequencing information is the preferred method for classifying organisms (Olsen, et al., 1986). Sequences can be rapidly obtained and stored in a database to allow future comparisons. Ribosomal RNA or DNA sequence information is used when characterizing microbial populations. rRNA is required for protein synthesis, and this function has been maintained throughout evolution. There are conserved 16S rRNA sequences among all organisms, and these conserved regions are important in primer development for amplifying the genes encoding rRNA, or rDNA. However, there are enough dissimilar regions to allow meaningful comparisons between sequenced samples. Also, rRNA's are

particularly useful in classifying organisms because the differences among them seem to be due to evolution and not lateral transfer (Olsen, et al., 1986) .

Although both 5S and 16S rRNA sequences have been used for phylogenetic analysis, the 16S subunit is preferred because it has more nucleotide variation. Comparisons of 16S rRNA or their genes began with partial sequence analysis, but now complete sequencing is possible (Olsen, et al., 1986).

Pace began using rRNA from organisms obtained from the environment rather from cultured isolates. He cloned and sequenced 16S rRNA genes from the environment and designed probes based on the sequences to quantitatively study the environment. The rRNA was useful to distinguish between organisms and for probe use because the high density of rRNA in cells made it readily visualized when hybridized (DeLong, et al., 1989, Woese, 1994)

Probes

A few years after initial use of 16S rRNA probes to study environmental samples, Stahl and colleagues (1988) used 16S rRNA probes to study the microbes in the rumen before, during, and after addition of monensin to the diet. This was one of the first studies to utilize molecular, rather than traditional culture-based, methods of studying the rumen ecosystem. Their main objective was to study whether changing microbial populations or changing microbial metabolism was responsible for the effects of monensin. The study investigated multiple strains of *B. succinogenes* and *L. multiparious*-like organisms as representatives of both Gram (-) and Gram (+) bacteria, respectively, and their response to the ionophore antibiotic monensin. They collected samples from a rumen-fistulated cow every day during the study (Stahl, et al., 1988).

Because monensin acts on Gram (+) bacteria, workers expected measurable shifts in *L. multiparious* upon exposure to the antibiotic. *L. multiparious* numbers did decrease, but not quickly and the proportion later increased. After monensin was removed, the probe detected more *L. multiparious* than it had previously – nearly twice as many as the largest detection. However, this spike lasted less than a week. Although there were two time periods within monensin treatment during which *L. multiparious* increased, the overall trend of its population decreasing with monensin exposure and increasing upon withdrawal was expected (Stahl, et al., 1988).

The authors conclude that, based on data from the two groups of bacterial species, changes in the microbes upon addition of monensin may be due to metabolic and not population shifts. However, possible problems with this study include the method of sampling ruminal contents and the limited number of bacteria investigated. The authors sampled the ruminal contents from the liquid phase below the mat, thus not obtaining a representative sample from the entire rumen by leaving out the mat. The authors tracked only two species and conclusions about the behavior of the rest of the population cannot be drawn. Finally, as the authors noted, the study did not last long enough to make more definitive conclusions (Stahl, et al., 1988).

Briesacher and coworkers (1992) also used probes to monitor the response of ruminal bacteria to feeding. One of their objectives was to study total 16S rRNA and *B. succinogenes* concentration as well as cell numbers with respect to time after feeding and location in the rumen. Samples were taken three times on the eleventh day of the trial, 0, 3, and 9 hours after the morning feeding. Samples were collected from both the mat and

the fluid phase of the digesta. Throughout the day, rRNA concentration of the mat increased, while there was no change in the rRNA of the fluid (Briesacher, et al., 1992).

The authors also performed cell counts throughout the day to investigate a possible relationship to the ratio of rRNA:RNA. Because they found no correlation between the ratio and the cell numbers, they concluded that rRNA can be used to study bacterial population shifts because rRNA fluctuated during metabolic shifting and not with changes in cell numbers (Briesacher, et al., 1992).

PCR

PCR is commonly one of the first steps in studying the populations of microbial communities. It provides numerous copies of sequences of interest, which allow for cloning, sequencing, or digestion of the amplicon. Although it is extremely useful, it does have limitations such as amplification inhibition, differential amplification, and artifact formation such as chimeras. Samples from microbial communities may have contaminants that could inhibit amplification. Contamination by a foreign microbe can be a serious problem when using universal bacterial primers, so sterility is imperative and using both positive and negative controls in the reaction is necessary. Also, microorganisms can have different rRNA operon numbers that are independent of their genome size. Therefore, quantification of the microbes in the population becomes difficult without knowing the operon number of the species of interest. Finally, suitable primer choice is a crucial step in PCR. There are obvious problems with using the same set of primers for both archaea and bacteria, as they are two of the three domains of life. Thus, their 16S rDNA sequences are dissimilar enough that at least two primers sets are

necessary to study a population with organisms present from both domains (Wintzingerode, et al., 1997).

Clone Libraries

Not only can 16S rRNA sequences be used for generating probes, but they can also be used to create clone libraries. Whitford and coworkers (1998) were the first to use this technique to study rumen microorganisms in the dairy cow. They did so by extracting DNA from ruminal samples, amplifying the 16S rDNA, cloning the amplicons, and analyzing their sequences. They found that across libraries, most clones clustered with *Prevotella*, warranting future investigation of those species and strains. The effect of PCR cycle number was tested and the lower cycle number resulted in more diverse clones. Clones were constructed from both the mat and the fluid sections of the rumen. The mat section had an even distribution of Gram (+) and Gram (-), while the fluid had much more Gram (-) than Gram (+) organisms. The Gram (-) sequences of either library clustered within the Cytophaga-Flavobacter-Bacteroides (CFB) phylum, most clustering with *Prevotella ruminicola*, and the Gram (+) sequences clustered within the low G+C gram (+) (LGCGPB) phylum and were most similar to *Clostridia* (Whitford, et al., 1998). Constructing clone libraries provides the ability to design probes and primers from the sequences to quantify and qualify populations (Whitford, et al., 1998).

Tajima and coworkers also constructed clone libraries of rumen content and found that most of their sequences were in the LGCGPB phyla, a quarter in the CFB phyla, and some in the Spirochaetes. They also found differences between the solid and liquid phases of the rumen, reporting more LGCGPB from the mat. The authors discussed not finding any sequences matching *Fibrobacter succinogenes* and could not explain its

absence. This study highlights problems faced with all clone library experiments, such as PCR bias, inefficient cloning into plasmids, and randomly picking up clones to sequence. The highest cloning efficiency was 80% (Tajima, et al., 1999).

The same group later constructed three more libraries to study changes in ruminal populations in response to dietary change from a hay to a high-grain diet. Because high producing cows are often switched to a high-grain diet, it is a common adaptation for ruminal microbes to undergo. After a four week adaptation to the diet, samples used for the library construction (0, 3, and 28 days into the adaptation) were chosen based on VFA concentration and animal characteristics throughout the four weeks (Tajima, et al., 2000). The authors' phylogenetic analysis was consistent with previous culture-based work, and also revealed previously unknown microbes (Tajima, et al., 2000).

Several findings of this study are consistent with culture-based studies, such as an increase in *Ruminococci* spp. and an increase in lactate producing or lactate using bacteria with increasing grain in the diet. However, many sequences analyzed belong to unknown organisms and highlight the need for more extensive phylogenetic work on deposited sequences. The authors reiterated Whitford and colleagues' (1998) discussion of the need to use probes and primers based on new sequence data rather than data gleaned solely from culture methods (Tajima, et al., 2000).

Edwards and colleagues (2004) pooled data from these three clone libraries created from ruminal samples and also gathered information from the Genebank database as well as the ATCC and DSMZ culture collections to investigate if pooling data gives a more complete picture. One problem encountered during the analysis was having matches to bacteria that were sequenced from samples other than the rumen, such as pig

gut and the human oral cavity. This can happen when using a complete database for identifying organisms from one niche (Edwards, et al., 2004). These bacteria could inhabit both these places and the rumen, or they could be separate bacteria that share similar 16S rDNA sequences. Therefore, using molecular methods is a tremendous leap from traditional culturing techniques, but final conclusions about a particular organism cannot be made without cultivating it. Classifications will continue to change as previously uncultured organisms are cultivated and provide phenotypic and other data (Edwards, et al., 2004).

Possible biases of using clone libraries were investigated. Tremendous diversity was noted across the three libraries, as the individual libraries were likely not large enough to adequately account for the microbial diversity of the populations (Edwards, et al., 2004).

Clone library studies investigating archaea have focused on methanogens, and one such study used samples from the rumen of sheep (Wright, et al., 2004). Investigating primer choice was not an aim of the study, but the libraries helped reveal the importance of it. The purpose of this study was to investigate the diversity of methanogens, an objective that was not met because the primers used did not amplify many of the archaeal microorganisms. In fact, the *Methanobrevibacter* group accounted for more than 90% of the identified clones (Wright, et al., 2004).

Other possible problems with this study include the sampling method. Only 50 milliliters of rumen fluid was collected from each sheep and there were two methods of collection for the three treatment groups. Fluid was collected from two groups via stomach pump, and collected from the other group via fistula. Collecting such small

samples from different areas of the rumen could have significantly affected the results (Wright, et al., 2004).

Skillman and colleagues (2006) studied the diversity of rumen methanogens as well as the effects of primer choice, as their effectiveness greatly affects the quality of the libraries and thus the organisms sequenced. By using two different primer pairs to create two libraries from the same sample of bovine rumen fluid, the bias created by primer choice was demonstrated. Primers greatly influence the composition and distribution of organisms revealed, and data on archaeal groups may be skewed because of poor primer choice. One primer pair, Arch f364/Arch r1386, produced sequences mostly within *Methanobrevibacter* spp. (Mbb), while sequences from another pair, 21f/958r were mostly within *Methanosphaera stadtmanae* (Msp). One benefit in using these primers is if the investigators are specifically interested in the Mbb and Msp groups. However, if the goal is to study the entire archaeal population, these primers are not suitable, so three new primers were developed for broader methanogen amplification. Developing broader primers is important for reducing cost and variability within a study (Skillman, et al., 2006).

Real-time PCR

Tajima et al. performed the first study that used real time PCR to quantify the microbial populations in the rumen (2001) by developing 13 primers for both qualifying and quantifying specific microbial species. The study investigated the changing bacterial species during dietary changes in cows by sampling the ruminal contents throughout the trial, and then purifying the DNA and performing both conventional PCR and real time PCR. Following the feeding and sampling protocol from their clone library experiment,

the diet was switched from hay to grain on day 0, and samples were taken from the rumen on days 0, 3, and 28 of the trial. Using both PCR and real time PCR added another dimension to the study. Species-specific primers allowed investigation of both the presence and amount of microbes on a species-dependent basis. The primers amplified less conserved sequences between species and more conserved regions within species so distinguishing between closely related species was possible. All of the primers amplified the targeted species on day 0 before the switch to the grain diets. The amount of *Fibrobacter succinogenes* decreased on day 3 and even more so on day 28, as did *Ruminococcus flaveflaciens* and *Eubacterium ruminantium*. *Steptococcus bovis* increased on day 3, but dropped below day 0 levels on day 28, and *Prevotella* spiked on day 3 and dropped on day 28. The authors compared their data with previous library experiments and found when using conventional PCR, *Fibrobacter succinogenes* may not amplify well, which may explain why rtPCR provided more data on that species (Tajima, et al., 2001).

Denman and McSweeney expanded on this work by designing primers for *Fibrobacter succinogenes* that reduced the amplicon size from 445 to 121 base pairs to improve amplification efficiency. They also reduced the size of the *Ruminococcus flaveflaciens* amplicon (Denman and McSweeney, 2006).

RFLP

Restriction fragment length polymorphism, or RFLP, is a genome-wide approach for identifying species based on what patterns and fragment lengths are produced after digestion with restriction enzymes. Because restriction enzymes cleave at specific sites of several nucleotides in length, just one nucleotide difference may be detected with this

method. Therefore, if two samples yield two fragments several thousand nucleotides in length, it could be because one nucleotide difference prevented the restriction enzyme from cleaving. Fragments of interest can be detected by using a Southern blot, so RFLP also utilizes the same principles of probe hybridization as previously discussed. This technique requires using probes, perhaps thousands, to locate the sequences or genes under investigation. This technique is commonly used for studying genetic diseases, as it provides a starting point for locating genes involved in disease.

Narayanan et al. (1997) used RFLP to investigate possible differences in *Fusobacterium necrophorum i*solated from liver abscesses, the ruminal wall, and the ruminal contents of eleven slaughtered cattle. The authors hypothesized that the bacteria present in liver abscesses come from the ruminal wall and not the ruminal contents because of the direct relationship between the number of liver abscesses and ruminal lesions. They used specific RFLP, ribotyping, which still utilizes DNA extracted from the whole genome, but the probe was cDNA of 16S and 23S rRNA from *E. coli.* The authors used a Southern Blot to investigate sequence similarities and classified strains as distinct if they had at least one different band appear on the blot (Narayanan et al., 1997).

The ruminal wall pattern matched that of the abscesses of the same cow in eight of the nine samples studies, but the patterns of the rumen contents never matched the patterns of either the wall or the abscesses. Finding a match between the rumen contents and the abscesses was not expected because just one strain can cause the abscess, and there are numerous strains of *F. necrophorum* in the rumen (Narayanan et al., 1997). Being able to study different strains of bacteria in various locations in the body helps investigators understand the development of liver abscesses and other conditions.

T-RFLP

In 1997, Liu et al. described a new approach for characterizing populations of microorganisms that builds upon the previously discussed molecular methods of DNA extraction, PCR amplification of 16S rDNA, restriction enzyme digestion, and comparisons of 16S rDNA sequences. The technique, Terminal Restriction Fragment Length Polymorphism (T-RFLP), is a rapid way to profile a community of microorganisms without relying on culture-based techniques. The first steps, DNA extraction and PCR amplification, are also used in the previously discussed clone libraries. The next step, restriction enzyme digestion, also is used in studying RFLP. The novelty of T-RFLP lies in its primer design. The forward primers are fluorescently labeled, so only the 5' terminal restriction fragments are analyzed. By only using the 5' restriction fragment, not only is the resolution of the gels greater, but also the analysis performed is drastically reduced (Liu, et al., 1997). As with all studies involving PCR, care must be taken in primer choice. As discussed with the clone libraries of methanogens, it is prudent to use a primer set to amplify bacteria and one for archaea because there are no universal primers that amplify each of the Eukarya, Bacterial, and Archaeal domains. The majority of 16s rDNA are similar enough to be amplified by a universal primer and the resulting terminal restriction fragments are so distinct that assessment of community diversity is possible. However, as discussed, the term universal to describe the primers is misleading. After searching the sequence database, the authors noted that their primers would not amplify archaeal 16S genes (Liu, et al., 1997). Another important decision in T-RFLP is choosing the restriction enzymes. Liu et al. used Pat Scan, a pattern-matching program, to aid in both their primer and

restriction enzyme choice. The restriction enzymes *Rsa*I, *Hha*I, and *Msp*I were chosen for having 4-base pair recognition sites and for yielding many diverse terminal restriction fragments (Liu, et al., 1997).

One of the biggest problems with the analysis of T-RFLP is using an incomplete database, but as more studies are conducted, more sequences deposited, and more phylogenetic work completed, this problem should lessen. The fragment length measurements of fragments less than 600 base pairs were accurate within 2 base pairs, but more leeway may need to be given when identifying species from fragments greater then 600 base pairs (Liu, et al., 1997)

Since the development of T-RFLP, many groups have used the technique to study communities of microorganisms, and it has been shown to be a specific, reliable, and reproducible method for studying such populations (Dunbar, et al., 2001). Although becoming an established method in microbiology, it has not been used to profile populations in the rumen.

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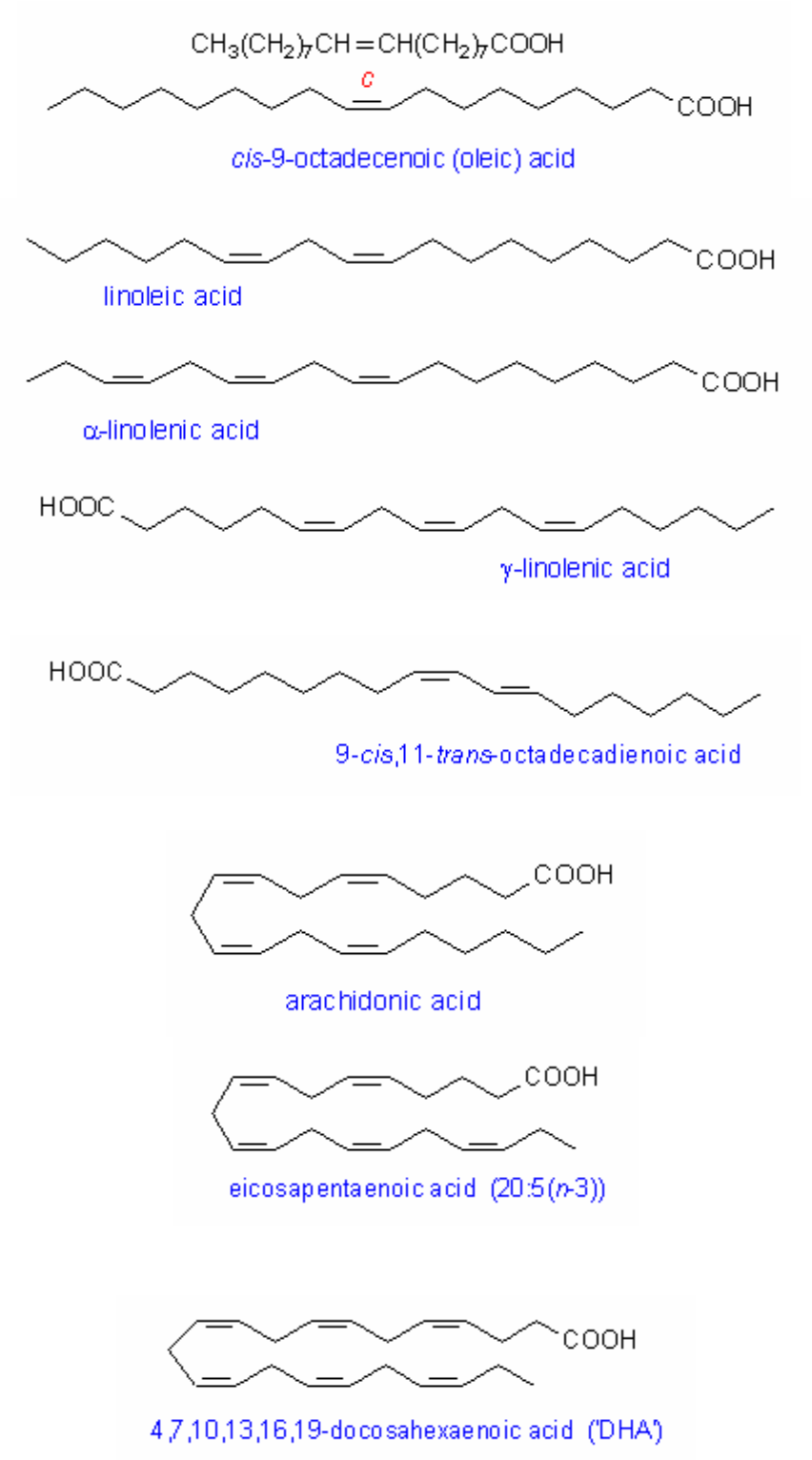

Figure 1. Backbone structures of oleic acid (C18:1), linoleic acid (C18:2), α and γ-linolenic acid (C18:3), CLA, AA, EPA, and DHA.

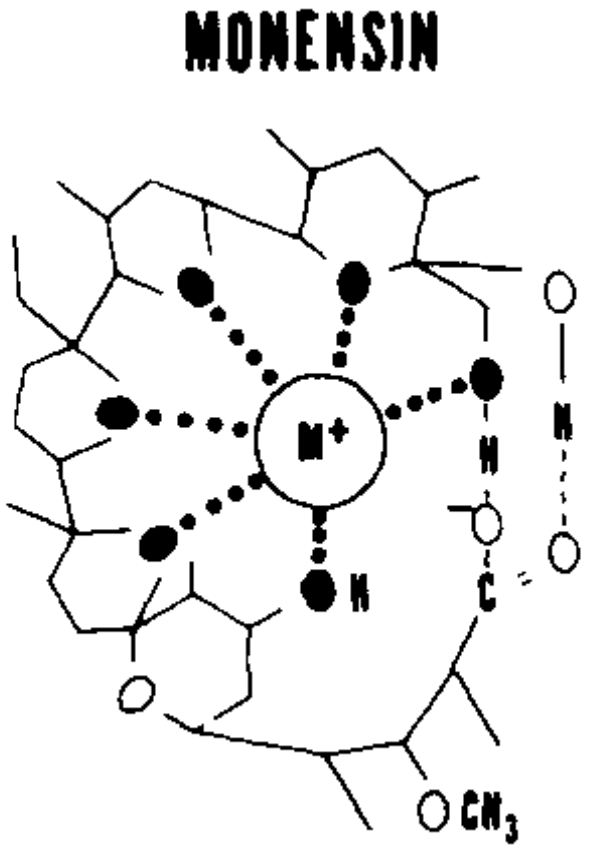

Figure 2. Structure of Monensin.

CHAPTER 3

EFFECT OF HIGH DHA ALGAL OIL ON MICROBIAL BIOHYDROGENATION

ABSTRACT

The objective of this study was to investigate the potential of marine algae to enhance incorporation of long-chain polyunsaturated fatty acids (LC-PUFA) into milk. Consumption of LC-PUFA has proven to improve human health by reducing the risk of heart disease, obesity, and diabetes and their incorporation into milk would provide a way to increase omega-3 (n-3) consumption. Mixed cultures of rumen microbes were incubated in artificial fermentors for a total of 9d. The trial was replicated three times within one run. The fermentors were gradually adapted over 4 d to a diet with a 50:50 forage:concentrate ratio from an all-forage diet. One fermentor served as a control (C) and only received the basal diet of corn silage and concentrate mix. Two fermentors served as treatment and received the basal diet plus supplemental algal oil. One received the oil in its extracted (E) form while another received it intact (I). Fermentation variables measured were volatile fatty acids (VFA), methane production, culture pH, ammonia (NH_3), and long-chain fatty acids (LCFA). Compared to C, addition of the algal oil tended to lower acetate ($p<0.10$), increase propionate ($p<0.03$), and decrease the A:P ratio ($p<0.03$). The algal oil also lowered methane production ($p<0.002$), and raised culture pH ($p<0.001$). There were differences in VFA production between E and I, as I had lower acetate ($p<0.05$), higher propionate ($p<0.005$), and lower A:P ($p<0.01$) than E. Methane production ($p<0.001$) and culture pH ($p<0.03$) were lower in I than E. Control cultures had significantly greater C18:0 when compared with cultures receiving the algal oil. Algal oil, either intact or extracted, resulted in substantial quantities of LC-PUFA in ruminal cultures, reflecting the greater concentrations of those fatty acids in the oil. Biohydrogenation was greater in C when compared to either E or I.

A second in vitro batch culture study was conducted to monitor the effects of the C, E, and I diets on fatty acid biohydrogenation over time. A 0.5 g substrate was placed into 200 ml bottles and 30 ml of a mixture of rumen inoculum and artificial saliva (1:2 ratio) were added. Bottles were capped and placed in a water bath at 39^{o}C for 0, 2, 4, 6, and 24hr. Three bottles per treatment were used to provide for additional replication (n=3). The differences in the percentages of the fatty acids across treatments at 24hr were similar to those found in the continuous culture study. However, the quantities of the fatty acids revealed the same amount of C18:2 across treatments with increased *cis*-C18:1 and *trans*-C18:1 in the E and I. Little biohydrogenation of the LC-PUFA was observed, and DHA decreased by an average of 2.7 mg. Our data suggest that biohydrogenation of the LC-PUFA in the algal oil was not extensive. There was an accumulation of the 18:1 *cis* and *trans* isomers in cultures receiving E and I and it seems that the DHA and DPA in the algal oil interfered with the terminal step of 18:1 biohydrogenation to 18:0.

INTRODUCTION

Much research has been aimed at improving the fatty acid profile of milk so that the proportion of saturated fat is decreased and that of unsaturated fatty acids is increased (Jenkins and McGuire, 2006). Recent efforts have focused on increasing the concentration of LC-PUFA, particularly that of omega-3 (n-3) FA, in milk. These fatty acids have been shown to enhance infant growth and visual and cognitive development and reduce the risk of cardiovascular disease, autoimmune disorders, type 2 diabetes, hypertension, rheumatoid arthritis, and certain cancers (Connor, 2000, Simopoulos, 1999).

In spite of these health benefits, intake of n-3 fatty acids from typical diets is usually low and the n-6 intake is high. This raises the n-6 to n-3 ratio to about 20-30:1, up from the healthier 1-4:1 (Simopoulos, 1999). The skewed n-6 to n-3 ratio perpetuates metabolic disorders by increasing pro-inflammatory products, therefore promoting thrombi and atheromas formation, leading to allergic and inflammatory disorders, and contributing to cell proliferation. Increased n-3, on the other hand, produces hypolipidemic, antithrombotic, and anti-inflammatory effects (Simopoulos, 1999).

Besides egg yolk, chicken, and oily fish, human milk and supplemented food are the only foods in the typical US diet that are a good source of DHA. As of 2004, no infant formula or baby foods in the United States contained fish (Hoffman, et al., 2004). Mothers are discouraged from eating too much fish because of the risk of metal contamination and, like children, may consume very little or no fish (Oken, et al., 2003). This makes it imperative to provide sources of n-3 PUFA apart from fish or fish oil

supplements. Because of the already beneficial effects of consuming dairy for mothers and children, enrichment of milk with healthful fatty acids would help provide a portion of the recommended daily LC-PUFA allowance.

Numerous factors affect the fate of unsaturated fatty acids in the rumen, and the most extensively studied are those that affect microbial activity or biohydrogenation (Jenkins, 1993). Biohydrogenation occurs after hydrolysis and is a process during which microbial enzymes add hydrogens to unsaturated fatty acids, making them more saturated (Jenkins and McGuire, 2006). Extensive biohydrogenation of unsaturated fatty acids in the rumen is implicated as a major process that determines the profile of fatty acids supplied to the mammary gland. Thus, strategies to enhance milk fat LC-PUFA would involve increasing rumen outflow of these fatty acids and their subsequent incorporation into milk fat. The fatty acids may have to be delivered to the rumen in a protected form, such as the raw plant material, rather than the extracted oil.

Numerous groups have attempted to use fish oil to increase LC-PUFA in the milk (AbuGhazaleh, et al., 2002, Donovan, et al., 2000, Lock and Bauman, 2004, Palmquist and Griinari, 2006). Several studies have incubated rumen fluid with fish oil or pure LC-PUFA to investigate their fate in the rumen, including their possible effects on and involvement in biohydrogenation (AbuGhazaleh and Jenkins, 2004a, AbuGhazaleh and Jenkins, 2004b, Dohme, 2003, Gulati, et al., 1999). These experiments have shown that both the oil concentration in the fluid and the fatty acid concentration in the oil influence fatty acid metabolism (AbuGhazaleh and Jenkins, 2004a)

In the first study that fed marine algae, there was incorporation of LC-PUFA into the milk, and the algae protected with xylose had even greater incorporation (Franklin, et

al., 1999). The objectives of the experiment outline herein were to 1) monitor the changes in rumen fermentation in response to algal oil, including biohydrogenation, and to 2) investigate possible protection from biohydrogenation by the algal oil in its intact form. It was hypothesized that including algal oil in its intact form in contrast to algal oil in its extracted form would impart greater degree of inertness in the rumen and thus allow for greater incorporation into milk fat.

MATERIALS AND METHODS

Continuous Culture Experiment

Rumen fluid collection and fermentor set-up

Rumen fluid was collected from a ruminally fistulated non-lactating Holstein cow fed a predominantly forage diet and housed at the North Carolina State University Dairy Educational Unit. The care and use of the fistulated animal study was approved by the North Carolina State University Institution of Animal Care and Use Committee. Approximately seven liters of fluid were sampled from various sections in the rumen using a manual hand held pump and, along with a handful of rumen digesta, placed in a sealed, preheated cooler and transported to the laboratory. Once in the lab, the fluid was filtered through double-layered cheesecloth, mixed thoroughly, and 700 mL were added to each of six dual-flow fermentors.

Several hours before the addition of ruminal inoculum, the fermentors were purged with CO_2 to displace any oxygen, heated to 39°C, and the saliva line was primed. Throughout the trial, the water bath maintained the fermentor temperature at 39°C, CO_2 flow was set at a constant rate at 20.0 ml/min, and artificial saliva was delivered with a

precision pump at 1.17mL/min that resulted in a fractional liquid dilution rate of 10%/hr. The fermentors were mixed continuously at 10 rpm.

Experimental Protocol

Dietary Treatments

The trial was replicated three times within one run, as eight fermentors were used, two served as controls, three were treated with the I diet, and three were treated with the E diet. The first two days were an adaptation period, during which the fermentors received a 100% forage diet consisting of alfalfa pellets twice daily for a total of 18 g of feed (DM basis) per day. On d3, the fermentors began a gradual adaptation to the experimental diets, as their diets consisted of 80% alfalfa hay and 20% experimental diets on d3 and 40% alfalfa hay and 60% experimental diets on d4. All fermentors received 100% experimental diets from d5 through d9.

Diet mixing

The fatty acid compositions of the diets are shown in Table 1. Alfalfa hay used in this experiment consisted of pellets and was purchased from a local feed store. The intact algal oil (diet I), extracted algal oil (diet E), and fat free biomass were all provided by Martek Biosciences (Winchester, KY). Diets were mixed with a Blakeslee Food Cutter (Model FC19; Ann Arbor, Michigan). For all diets, corn silage was mixed first, followed by the cottonseed hulls, and then the concentrate mix. The control diet (C) received no other ingredients. For the I diet, the intact algal oil was added next, and for the E diet, the fat free biomass and then the extracted algal oil were added. Each ingredient was mixed for one minute.

Sample collection and analytical procedures

Sample Collection

Methane and pH measurements were taken everyday of the trial. Samples for VFA, LCFA, and ammonia were obtained two hours after feeding on d7, d8, and d9. Prior to sampling culture contents, the speed on the automatic stirrers was increased to thoroughly mix fermentor contents. A pipette with a large opening was used to obtain a homogenous sample. Samples for LCFA also were taken from the overflow. The effluent was collected in a container on ice for 24 hours and was thoroughly mixed before sampling.

Methane and pH

Methane and pH were recorded everyday of the trial. Methane gas measurements were obtained by filling a gas-tight syringe (Hamilton Co., Reno, NV) with 10µl of fermentor headspace gas and directly analyzed using gas chromatography (model CP-3380; Varian; Walnut Creek, CA) according to Fellner, et al. (1997). Methane measurements were made at -1, 1, 2, 3.5, 5, and 6.5h after feeding. The pH was continuously monitored by a pH probe (Cole Parmer; Vernon Hills, Illinois) suspended in the rumen fluid and recorded at the time of methane analysis. Hourly methane and pH values were analyzed by day.

VFA and Ammonia

Two hours after feeding on d7, d8, and d9, two 1ml samples were obtained for VFA and ammonia analysis. The samples for ammonia were immediately stored at -70°C. The VFA samples were immediately processed by suspending in 200µl metaphospohoric acid in a 25% solution with 2 ethyl butyric acid as an internal standard

and centrifuging in a tabletop centrifuge (model Micromax; International Equipment Co.; Needham Heights, MA) at 21,000g for 10 minutes. The samples were then stored at -70°C until subsequent analysis. Ammonia was analyzed by a colorometric procedure (Beecher and Whitten, 1970) and the VFA were analyzed by gas liquid chromatography (model CP-3380; Varian; Walnut Creek, CA) using a fused silica capillary column with 30m x 0.25mm x 0.25µm film thickness (Nukol; Supelco Inc., Bellefonte, PA) according to Fellner, et al. (1997).

Fatty Acids

Two hours after feeding the morning feeding on d7, d8, and d9, 5mL samples LCFA analysis. Fatty acids were extracted using the Bligh and Dyer method (1959), methylated by the procedure outlined by Kramer and colleagues (1997) and analyzed by gas liquid chromatography (model CP-3380; Varian; Walnut Creek, CA). The column used was a fused silica capillary column (Nukol; Supelco Inc., Bellefonte, PA) with 100m x 0.25mm x 0.2µm film thickness. The injector temperature was 230°C. The oven temperature was held at 70°C for 4 minutes and then increased 13°C per minute until reaching 175°C, where it remained for 27 minutes. It then increased 8°C per minute to 215°C, where it remained for 45 minutes, for a total time of 89 minutes. The flame ionized detector (FID) temperature was 250°C.

Statistics

All data were analyzed using the Proc Mixed procedure of SAS. The random variable was the replicate. Orthogonal contrasts were performed between control (C) and algal oil (A) and extract (E) vs intact (I) samples. Significance is reported at $p < 0.05$.

Batch Culture Experiment

Rumen fluid collection and batch culture set-up

The protocol for rumen fluid collection was similar to that described above for continuous culture. Diets fed were also the same as described above (Table 1). Once in the lab, the fluid was filtered through double-layered cheesecloth and mixed with previously prepared artificial saliva in a 1:2 ratio. Before the addition of rumen fluid and buffer mixture, 0.5g of feed (DM basis) were added and the bottles were purged with CO_2 to displace any oxygen. Then 30 mL of the fluid and buffer mixture were added, the bottles were again purged with CO_2, capped, and placed in a water bath preheated to 39°C.

Experimental Protocol

Dietary Treatments

Sixteen bottles were used per replicate. One served as a negative control, as it only contained 30mL of the rumen fluid and buffer mixture. The other 15 bottles were split into 3 treatment groups, and each treatment received 0.5g (DM basis) of the C, E, or I diet. One bottle from each treatment was collected at 0, 2, 4, 6, and 24 hr after inoculation. The experiment was replicated 3 times.

Sample collection and analytical procedures

Fatty Acids

At 0, 2, 4, 6, and 24 hours, one bottle from each treatment was removed from the water bath, the cap was sealed, and the bottle was cooled to 4^oC. One mL of C19:0 at 2.5mg/mL was added as an internal standard and the entire contents of each bottle were extracted using the Bligh and Dyer method (1959) with some modifications. To the

30mL of rumen fluid and buffer mixture, 9mL of dH_2O, 3mL of 6N HCl, and 60mL of 1:1 chloroform:methanol were added, swirled to mix, and left to sit for several hours. The bottom layer was removed, 10mL of 1:1 and 15mL of chloroform were added, swirled to mix, and again left to sit. The bottom layer was removed and the procedure proceeded as normal. The extractions were then methylated by the procedure outlined by Kramer (1997) and analyzed by gas liquid chromatography (model CP-3380; Varian; Walnut Creek, CA).

Statistics

All data were analyzed using the Proc Mixed procedure of SAS. The random variable was the replicate. Significance is reported at $p < 0.05$.

RESULTS

Continuous culture experiment

Fermentation parameters

Table 2 shows the effects of the C and experimental A diets on total and individual VFA concentrations. The table also shows comparisons between the two A diets, E and I. Total VFA (mM) did not differ between C and A or between E and I, as it averaged 29.8 mM for C, 34.1 mM for E, and 30.1 mM for I. Also not differing between C and A or between E and I were propionate (mM) and isovalerate (mM). Both acetate and butyrate mM were the same between C and A, but the two differed among A, with the E resulting in higher acetate ($p<0.03$) and butyrate ($p<0.008$). The valerate concentration was lower in C than A ($p<0.01$) as well as lower in E than I ($p<0.007$). E

resulted in higher valerate concentration than either C or I. Isobutyrate, however, was higher than A ($p<0.001$), but did not differ between E and I.

The molar percentages of individual VFA are also presented in Table 2. Acetate (A) mol/100mol did not differ between C and A, but was lowered with I treatment compared to E ($p<0.05$). Propionate (P) was increased with A treatment when compared to C ($p<0.03$) and was increased more with I than with E ($p<0.005$). In fact, the increase with I may be the cause for the increase of A over C. Because of a trend ($p<0.10$) for the algal oil diet to decrease acetate over control and the significant ($p<0.03$) increase in propionate due to the algal oil diet, the treatments lowered the A:P ratio ($p<0.03$). As with the changes in propionate, the decrease in A:P may be due to the I treatment lowering A:P even more than the E treatment ($p<0.01$).

The molar percentages of butyrate and isovalerate were unchanged from C by A treatment, but the mol/100 mol of isobutyrate and valerate were both changed by A ($p<0.003$ and $p<0.02$), but did not differ between E and I. Isobutyrate mol/100 mol was lowered by A and valerate mol/100 mol was increased by A treatment.

Table 3 shows the effects of A on methane, culture pH, and ammonia concentration, as well as the effects of the different A treatments, E and I, on those parameters. Ammonia mg/100mL did not differ with A compared to C, and neither E nor I changed the concentration. Treatment A lowered daily methane production from 22.1 mmol/day ($p<0.002$), and I lowered it down to 11.8 mmol/day, even more than E that was at 18.9 mmol/day ($p<0.001$). Culture pH was 6.0 in C fermentors and was increased with A ($p<0.001$). Treatment I raised the pH of the culture to 6.2, and E raised it more than I ($p<0.03$) to 6.3.

Fatty acids, percentage of total fat

The effects of the treatments on the fatty acid profile of the fermentors are shown in Table 4. None of the percentages of fatty acids differed between the E and I treatments, but most of the FA proportions were significantly different from the C and the A treated fermentors. The percentage of C16:0 was greater after A treatment compared to C ($p<0.01$). The percentages of both C18:0 and *cis*-C18:1 were greater in C than A ($p<0.003$ and $p<0.02$, respectively), but *trans*-C18:1 did not differ between treatments.

The percentages of C18:2, C18:2 *cis*-9, *trans*-11, and C18:2 *trans*-10, *cis*-12 were all significantly higher in C than with A. The percentages of AA, EPA, DPA, and DHA were also all higher in A than in C.

Table 5 shows the effects of treatment on the fatty acid profile of the overflow from the fermentors. As with the fermentors, most of the FA proportions were significantly different between C and A. There was only a trend ($p<0.09$) for the percentage of C16:0 to be greater after A treatment compared to C. The percentages of both C18:0 and *cis*-C18:1 were greater in C than A, but *trans*-C18:1 did not differ ($p>0.10$) between treatments.

The proportion of C18:2 was higher in C than A overflow ($p<0.02$) and higher in E than I overflow ($p<0.02$). Unlike the fermentors, the percentages of the CLA isomers C18:2 *cis*-9, *trans*-11 and C18:2 *cis*-10, *trans*-12 did not differ between C and A overflow. As with the fermentors, the percentages of AA, EPA, DPA, and DHA were all higher in the overflow from the A treated fermentors than in C.

Batch culture experiment

Fatty acids, percentage of total fat

The percentages of individual fatty acids in batch culture are shown in Table 6. At 0hr, the percentages of C16:0, AA, EPA, DPA, and DHA were all significantly higher in E and I than in C ($p<0.01$) and the percentage of Others was also higher in A than C ($p<0.05$). C18:0, *trans*-C18:1, *cis*-C18:1, and C18:2 all were higher in C than E or I ($p<0.01$). The proportions of non-conjugated C18:2 (*cis, trans*), C18:2 *cis*-9, *trans*-11, and C18:2 *trans*-10, *cis*-12 did not differ across treatments.

At 2hr, C16:0, AA, DPA remained higher in A ($p<0.01$) and DHA and Others also stayed higher in A ($p<0.05$). C18:0, *trans*-C18:1, *cis*-C18:1, C18:2, C18:2 *cis*-9, *trans*-11, and C18:2 *trans*-10, *cis*-12 were all higher in C than E or I ($p<0.01$). EPA was highest in I, followed by E, and lowest in C ($p<0.01$). The proportions of non-conjugated C18:2 (*cis, trans*) remained the same across treatments.

At 4 hr, C16:0, DPA, and DHA remained higher in both I and E than C ($p<0.01$), as did Others ($p<0.05$). Both AA and EPA were highest in I, followed by E, and lowest in C ($p<0.05$). C18:0, *trans*-C18:1, C18:2, C18:2 *cis*-9, *trans*-11, and C18:2 *trans*-10, *cis*-12 remained higher in C than in E or I ($p<0.01$). The proportion of *cis*-C18:1 was highest in C, followed by E, and lowest in I ($p<0.05$). The proportions of non-conjugated C18:2 (*cis, trans*) still did not differ across treatments.

At 6hr, C16:0, AA, DPA, DHA, and Others were higher in both I and E than C ($p<0.01$). EPA remained highest in I, followed by E, and lowest in C ($p<0.05$). C18:0, *trans*-C18:1, *cis*-C18:1, C18:2, C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 were all

higher in C than in E or I ($p<0.01$). The proportions of non-conjugated C18:2 (*cis, trans*) still did not differ across treatments.

At 24hr, C16:0, EPA, DPA, and DHA were higher in both I and E than C ($p<0.01$). AA remained highest in I, followed by E, and lowest in C ($p<0.01$). C18:0, *trans*-C18:1, *cis*-C18:1, C18:2, were all higher in C than in E or I ($p<0.01$), as well as the proportions of non-conjugated C18:2 (*cis, trans*) ($p<0.05$). C18:2 *cis*-9, *trans*-11, C18:2 *trans*-10, *cis*-12, and Others did not differ across treatments.

Fatty acids, mg of total fat

The proportions of the individual fatty acids in each treatment in the batch culture are presented in Table 7. At 0hr, the amount (mg) of all of the LC-PUFA (AA, EPA, DPA, and DHA) were higher in E and I compared to C ($p<0.01$), as was the amount of C16:0 ($p<0.05$). *Trans*-C18:1 was higher then I than either C or E ($p<0.05$). None of the weights of the other fatty acids differed across treatments.

At 2hr, the amount of C16:0, all of the LC-PUFA and the Others were significantly greater in E and I than C ($p<0.01$), as was the amount of *trans*-C18:1 ($p<0.05$). The other fatty acids did not differ in the three treatments.

At 4 hr, the amount of C16:0, DPA, DHA, and the Others remained greater in E and I compared to C ($p<0.01$), and the mg of *cis, trans* C18:2 was also greater in E and I ($p<0.05$). Both AA and EPA weights were greatest in I, followed by E, and lowest in C ($p<0.01$ and $p<0.05$, respectively). The remaining fatty acids were not significantly different in C, E, or I.

There were more differences in the mg of the fatty acids at 6hr. C16:0, *cis, trans* C18:2, and DHA all remained higher in E and I than C ($p<0.01$). C18:2 *trans*-10, *cis*-12

was significantly different for the first time, as it was greater in C than in E and I ($p<0.05$). AA, DPA, and the Others were all greatest in I, followed by E, and were lowest in C ($p<0.05$), and EPA followed the same pattern at greater significance ($p<0.01$). The weights of the other fatty acids were similar across treatments.

The most differences between the mg of the fatty acids were observed at 24hr. The amount of C16:0, EPA, DPA, DHA, and the Others were all greater in E and I than C ($p<0.01$). Both *cis*, *trans* C18:2 and AA were highest in I, followed by E, and lowest in C ($p<0.05$). C18:0 was highest in C, followed by E, and lowest in I ($p<0.05$). C18:2 *trans*-10, *cis*-12 remained higher in C than E or I ($p<0.05$). For the first time, there were differences in *trans*-C18:1 and *cis*-C18:1, as they were both higher in I and E than C ($p<0.01$ and $p<0.05$, respectively). The remaining fatty acids, C18:2 and C18:2 *cis*-9, *trans*-11, did not differ across treatments.

DISCUSSION

Continuous culture fermentation parameters

There were metabolic differences between C and A, as methane and A:P decreased and pH increased in A. Based on the fermentation data, it appears that an algal oil supplemented diet improves the efficiency of fermentation more than the control diet. The intact form of the oil shows even more energetically favorable effects on fermentation.

There were no significant changes observed in VFA production, which is consistent with previous *in vitro* and *in vivo* studies on fish oil supplementation (Dohme,

2003, Lee, et al., 2005, Wachira, et al., 2000), although changes in VFA production have been observed (Palmquist, et al., 2004).

There was a trend for A to lower acetate and A significantly increased propionate and lowered A:P. This is consistent with fish oil studies that showed similar effects on these VFA (Shingfield, et al., 2003 , Wachira, et al., 2000). However, the I diet showed an even greater decrease in acetate, increase in propionate, and decrease in A:P. Studies supplementing marine algae have focused on biohydrogenation and not fermentation (Franklin, et al., 1999, Sinclair, et al., 2005), so it is difficult to compare the I treatment to previous studies.

The mM of butyrate increased with E compared to I, and there was a trend for the mol/100mol of butyrate to increase with E over I. This is consistent with other studies that have shown fish oil supplementation to increase butyrate (Shingfield, et al., 2003). This suggests that the free oil and not the intact algal oil has effects on microorganisms that produce butyrate (Maia, et al., 2007).

No significant differences were found in ammonia concentration in any of the fermentors, which has been reported in fish oil studies (Lee, et al., 2005, Wachira, et al., 2000). There were differences in methane production, however, as A decreased it and I decreased it even more than E. The decrease in methane due to marine algae has not been extensively studied. However, decreases in methane due to free fish oil have been reported and it has been hypothesized that a decrease in methane production is in response to an inhibitory effect of fish oil on methanogens (Wachira, et al., 2000).

The culture pH increased with A, and E increased it even more than I. This is supported by reports of increasing pH upon both fish and algal oil supplementation

(Shingfield, et al., 2003 , Sinclair, et al., 2005). Other studies have not observed changes in pH after exposure to fish oil (Dohme, 2003, Lee, et al., 2005, Wachira, et al., 2000).

The hypothesis was that the I diet would protect the algal oil from biohydrogenation, and it was expected that both I and E would affect fermentation.

Fatty acid percentages in continuous culture

The higher percentage of C 16:0 in the A fermentors compared to C is likely due to its presence in the algal oil diets. The proportions of C18:0, *cis*-C18:1, C18:2, and CLA were higher in controls, which could indicate a decrease in biohydrogenation due to the presence of algal oil, whether in the intact or extracted form. A common observation upon fish oil supplementation is that BH is greatly affected by LC-PUFA concentration (AbuGhazaleh and Jenkins, 2004a).

There appeared to be limited biohydrogenation of LC-PUFA. This is consistent with a investigation of different supplements that found less biohydrogenation of marine algae than of fish oil by rumen microorganisms (Sinclair, et al., 2005). There are several theories for this, including the absence of enzymes specific for LC-PUFA, the LC-PUFA inhibiting of different enzymes with specific activities, or the inhibition of similar enzymes with different affinities (AbuGhazaleh and Jenkins, 2004a).

Inhibition of biohydrogenation or changes in the biohydrogenation pathway have been consistently observed during supplementation with fish oil, as indicated by an increase in the *trans*-11 C18:1 fatty acid and a smaller proportion of C18:0 compared to controls (AbuGhazaleh and Jenkins, 2004b). Because of its effects on fermentation and biohydrogenation, fish oil is often implicated in altering the microbial population in the rumen (Lee, et al., 2005, Palmquist and Griinari, 2006, Wachira, et al., 2000).

It has been suggested that fish oil is inhibitory and even toxic to certain rumen bacteria, notably cellulolytic bacteria (Maia, et al., 2007, Wachira, et al., 2000). Because the final step of C18 hydrogenation seems to be inhibited by fish oil (Gulati, et al., 1999, Lee, et al., 2005, Palmquist and Griinari, 2006), it may specifically inhibit linoleate isomerase (Shingfield, et al., 2003) or Group B bacteria (Gulati, et al., 1999, Palmquist and Griinari, 2006, Shingfield, et al., 2003).

Our results are consistent with an *in vitro* study that incubated a range of fish oil concentrations in sheep rumen fluid and showed the greatest biohydrogenation at 1mg/ml and the least at 5mgl/ml (Gulati, et al., 1999). The fatty acid concentration of the oil used was between 6 and 17% EPA and DHA. The authors noted that C18:2 biohydrogenation occurred and resulted in 18:1-*trans* and 18:0 formation even though EPA and DHA were only hydrogenated to a small extent.

Dohme and coworkers monitored the effects of oil concentration and fatty acid concentration on lipolysis and biohydrogenation (2003). Increasing concentration of the oils with the higher fatty acid percentage both increased the concentration of the unesterified fatty acids and decreased their biohydrogenation (Dohme, et al., 2003). Lipolysis seemed to depend most on the amount of oil in the fluid, while biohydrogenation depended on the concentration of unesterified fatty acids, as biohydrogenation was lower in the oil with the greater percentage of EPA and DHA (Dohme, et al., 2003). This could be another reason for low level of biohydrogenation in this study, as the algal oil supplement was very high in DHA.

Studies that have involved feeding soybean and fish oil to cows have shown an increase in biohydrogenation intermediates of C18:2 such as vaccenic acid (VA; *trans*-11

18:1) and CLA, which is striking, as fish oil contains little 18:2 and 18:3, yet appears to influence their metabolism (AbuGhazaleh and Jenkins, 2004b). Batch cultures of rumen fluid incubated with DHA for 24 hours showed that DHA appears to be at least one of the components in fish oil that affects biohydrogenation (AbuGhazaleh and Jenkins, 2004b). Docosahexaenoic acid caused a decrease in 18:0 and 18:1 formation compared to other treatments (AbuGhazaleh and Jenkins, 2004b), which is consistent with the current study.

Fatty acid percentages in batch culture

A major question remaining after the continuous culture study was why there was such a large difference in the proportion of C18:2 between the C and A, even though the amount of C18:2 in each diet was similar. The batch culture experiment was performed to address this issue.

Fatty acid quantities in batch culture

Of the three methods for analyzing the fatty acids in culture: percentages in continuous culture, percentages in batch culture, and quantities in batch culture, the quantities in batch culture appeared to give the most clear information on the changes in biohydrogenation. By having the total amount of each fatty acid throughout the 24 hour period, it is possible to see when the fatty acids began being metabolized or synthesized.

The quantities of the fatty acids over time indicate that little biohydrogenation of the LC-PUFA is occurring. Also, the conversion of C18:2 is not blocked by the presence of A, as the amount of the fatty acid does not differ across treatments at any time. However, A does seem to block complete biohydrogenation to C18:0, as this step only occurs in C. The presence of the very LC-PUFA in A may be altering the biohydrogenation pathways. Alternatively, although little biohydrogenation of the very

LC-PUFA is occurring, there may be formation of a potent isomer of DHA or of another LC-PUFA that might affect C18 fatty acids even at low amounts. The increases in *cis*-C18:1 and *trans*-C18:1 observed at 24hr is consistent with earlier studies (AbuGhazaleh and Jenkins, 2004b). It has been hypothesized that the increase in *cis*-11 C18:1 may be from chain elongation of C16:1 and the increase in *trans*-11 C18:1 could be from inhibition of hydrogenation to C18:0 (Palmquist and Griinari, 2006). Other theories on changes in lipid metabolism include the partial oxidation or hydrogenation of C20 and C22 fatty acids to C18 fatty acids (Donovan, et al., 2000) or the lack of specific isomerase and reductase enzymes for these very LC-PUFA (AbuGhazaleh and Jenkins, 2004b).

Of particular note that could not be determined by the continuous culture is that the mg amounts of C18:2 are not different across treatments at any time. In contrast, the percentages of C18:2 were higher in C than A in both the continuous and the batch cultures. The large difference in percentages is likely because the A diets have high proportions of the very LC-PUFA, which gives them a smaller percentage of C18:2 than C. Other important findings in the batch culture experiment are the higher amounts of *trans*-C18:1 in both E and I over C at 24hr and a higher amount of C18:0 in C over E and I. This suggests that the algal oil has an inhibitory affect on C18 biohydrogenation.

The fate of both DHA and EPA in batch cultures of rumen fluid have been investigated using pure forms of the fatty acids (AbuGhazaleh and Jenkins, 2004a). Several concentrations of DHA (0, 5, 10, 15, and 20 mg) and EPA (0, 5, 10, and 15 mg) were tested to determine the effect of level of fatty acid on biohydrogenation of DHA and EPA. The amount of DHA and EPA in the cultures decreased over time, with the

disappearance and the concentration of the added fatty acids exhibiting an inverse relationship. This can be used for comparison with the present study, as the E cultures were incubated with 15.5 mg DHA and the I cultures were incubated with 18.9 mg DHA, and a similar level of DHA biohydrogenation was observed.

It has been suggested that DHA and EPA could have been biohydrogenated, their carbon chain could have been shortened, or they could have been transformed into other C22:6 and C20:5 isomers, respectively (AbuGhazaleh and Jenkins, 2004a). Increases in unsaturated fatty acids, particularly C18:1, with addition of DHA and EPA raise the possibility that DHA and EPA could have influenced the unsaturated fatty acid concentration whether directly, via shortening of their carbon chain, or indirectly, by inhibiting the activity of the enzyme reductase of ruminal microorganisms (AbuGhazaleh and Jenkins, 2004a).

Concluding remarks

Algal oil altered the fermentation profile of continuous cultures by increasing propionate and decreasing A:P and methane production. Changes in these parameters, as well as an increase in pH, indicate shifts in the microbial population in response to supplemental algal oil.

There appeared to be limited biohydrogenation of LC-PUFA, particularly DHA, in both continuous and batch culture. Because the supplement was high in DHA, the high concentration of the fatty acid likely protected it from biohydrogenation. Because the fatty acid profile of both E and I were similar, it seems that the concentration of the fatty acid, rather than the presence of a protective substance, may be the most important factor in predicting the extent of biohydrogenation.

The biohydrogenation of DHA and other LC-PUFA appear inhibited by the algal oil in either its intact or extracted form, and the presence of the LC-PUFA appeared to alter the biohydrogenation of fatty acids 18 carbons in length. The batch culture showed C18:2 to be present in the same amount in all treatments after 24hr, but the absolute amount (mg) of C18:1 was greater in E and I compared to C; C18:0 was, however, greater in C compared to E and I. Given the lesser formation of 18:0 in E and I, it seems both those treatments inhibited biohydrogenation compared to C. The accumulation of 18:1 *cis* and *trans* isomers with E and I suggest inhibition at the terminal step leading to 18:0 formation.

The results from the quantities of fatty acids in batch culture reveal the limitation of using only percentages of fatty acids. Monitoring the absolute amounts (mg) of the fatty acids added another dimension to the study that allowed more accurate conclusions to be made about changes in biohydrogenation.

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Table 1. Fatty acid composition (% of total fatty acids) of the diets.

	Diet			
Fatty Acid	Control	Intact (I)	Extract (E)	Fat Free Biomass
C12	0.17	0.37	0.45	0.47
C14	0.34	10.01	11.00	13.16
i-15	0.00	0.01	0.00	0.00
C15	0.00	0.36	0.35	0.42
i-16	0.00	0.08	0.08	0.00
C16	20.34	26.19	28.41	28.92
C17	0.00	0.07	0.08	0.00
C18	3.14	0.93	1.08	0.91
C18:1 trans	0.77	0.10	0.09	0.69
C18:1 cis	20.52	3.70	4.71	0.54
cis-trans	0.00	0.12	0.14	0.06
C18:2	50.33	8.44	8.32	0.50
C20	0.41	0.14	0.14	0.00
C18:3n-3	3.54	0.66	0.76	0.04
AA (ARA)	0.00	0.66	0.52	0.50
EPA	0.00	1.77	1.49	1.88
DPA	0.00	11.03	10.89	13.22
DHA	0.00	26.96	27.36	35.27
Others	0.46	8.40	4.13	3.41

Table 2. Effect of treatment on total and individual VFA production.

	Treatments				Contrasts	
		Algae (A)				
	Control	Extract (E)	Intact (I)	SE	C vs. A	E vs I
Total VFA, mM	29.8	34.1	30.1	2.43	0.32	0.09
Individual VFA, mM						
Acetate (A)	16.7	18.9	15.9	1.40	0.62	0.03
Propionate (P)	7.3	8.6	8.5	0.81	0.13	0.89
Butyrate	3.3	4.0	3.2	0.28	0.24	0.008
Isobutyrate	0.4	0.3	0.3	0.03	0.001	0.73
Valerate	0.5	0.7	0.6	0.04	0.01	0.007
Isovalerate	1.7	1.9	1.7	0.12	0.48	0.09
Individual VFA, mol/100 mol						
Acetate (A)	56.3	55.5	52.9	1.33	0.10	0.05
Propionate (P)	24.3	25.1	28.1	1.02	0.03	0.005
Butyrate	10.8	11.6	10.7	0.64	0.52	0.11
Isobutyrate	1.3	0.8	0.9	0.13	0.003	0.50
Valerate	1.7	2.0	1.9	0.11	0.02	0.33
Isovalerate	5.8	5.5	5.6	0.44	0.62	0.83
A:P	2.3	2.2	1.9	0.13	0.03	0.01

Table 3. Effect of treatment on methane production, culture pH, and ammonia concentration.

	Treatments				Contrasts	
		Algae				
	Control	Extract (E)	Intact (I)	SE	C vs. A	O vs P
Methane, Mmol/d	22.1	18.9	11.8	2.01	0.002	0.001
Culture pH	6.0	6.3	6.2	0.06	0.001	0.03
NH_3, mg/100ml	23.2	23.3	24.5	1.19	0.54	0.27
Digestibility, %	44.8	48.8	40.5	3.01	0.94	0.007

Table 4. Effect of treatment on fatty acid composition (% of total fatty acids) of fermentors 2hr after AM feeding. (Values are means of last 3 days).

	Treatments				Contrasts	
		Algae				
Fatty Acid	Control	Oil	Powder	SE	C vs. A	O vs P
C12	0.3	0.3	0.4	0.08	0.88	0.59
i14	0.5	0.1	0.1	0.04	0.003	0.39
C14	0.9	7.1	7.9	0.87	0.004	0.36
i15	2.2	0.8	1.2	0.28	0.008	0.15
a15	1.3	0.5	0.6	0.11	0.004	0.55
C15	1.7	0.7	0.9	0.23	0.02	0.44
i16	2.5	1.2	1.3	0.50	0.08	0.81
C16	17.8	24.4	25.7	1.51	0.01	0.40
C17	0.7	0.2	0.2	0.01	0.0001	0.14
C18	35.2	3.1	3.4	3.73	0.003	0.93
C18:1 trans	8.7	7.0	8.0	1.19	0.36	0.41
C18:1 cis	8.7	5.1	4.0	1.04	0.02	0.32
cis, trans	1.5	0.5	0.6	0.10	0.003	0.44
C18:2	9.4	5.1	3.1	0.99	0.01	0.12
CLA 9,11	0.5	0.2	0.1	0.07	0.02	0.43
CLA 10,12	0.1	0.1	0.0	0.01	0.001	0.17
C18:3;C20:1	1.0	0.6	0.3	0.27	0.15	0.34
C20	0.7	0.2	0.3	0.03	0.0003	0.50
C22	1.8	0.2	0.4	0.67	0.10	0.76
AA	0.0	0.6	0.5	0.11	0.01	0.71
EPA	0.0	1.0	1.1	0.08	0.0007	0.16
DPA	0.1	10.3	10.5	0.92	0.001	0.80
DHA	0.0	24.8	23.8	2.05	0.001	0.64
Others	4.5	6.0	5.4	1.00	0.28	0.58

Table 5. Effect of treatment on fatty acid composition (% of total fatty acids) of the overflow 2hr after AM feeding. (Values are means of last 3 days).

	Treatments				Contrasts	
		Algae				
Fatty Acid	Control	Extract	Intact	SE	C vs. A	E vs I
C12	0.7	0.5	0.5	0.09	0.10	0.57
i14	0.7	0.1	0.1	0.06	0.002	0.82
C14	2.7	7.4	8.5	0.80	0.006	0.23
i15	11.8	4.8	4.4	1.74	0.02	0.83
a15	1.7	0.7	0.6	0.13	0.004	0.63
i16	8.6	5.5	3.6	1.52	0.06	0.26
C15	2.9	1.0	1.0	0.14	0.008	0.68
C16	20.3	24.4	26.0	2.12	0.09	0.47
C17	0.7	0.2	0.2	0.03	0.0004	1.00
C18	27.6	2.8	2.8	3.88	0.006	0.99
C18:1 trans	6.5	7.5	6.4	0.91	0.66	0.28
C18:1 cis	6.4	4.8	3.5	0.82	0.06	0.20
cis, trans	0.0	0.2	0.3	0.16	0.22	0.79
C18:2	3.9	3.1	1.8	0.31	0.02	0.02
CLA 9,11	0.0	0.0	0.0	0.02	0.49	0.87
CLA 10,12	0.0	0.0	0.0	0.01	0.62	0.35
C18:3;C20:1	0.0	0.3	0.2	0.03	0.002	0.16
C20	0.5	0.2	0.2	0.03	0.008	0.53
C22	0.0	0.1	0.1	0.08	0.49	0.97
AA	0.0	0.3	0.5	0.07	0.01	0.15
EPA	0.0	0.9	1.1	0.12	0.003	0.13
DPA	0.0	9.4	10.7	1.02	0.002	0.27
DHA	0.4	22.1	24.2	1.66	0.007	0.26
Others	4.4	3.8	3.4	0.51	0.28	0.55

Table 6. LCFA (% of total FA) for all treatments at 0hr (A), 2hr (B), 4hr (C), 6hr (D), and 24hr (E) in batch cultures.

A.	0hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	16.7[d]	23.6[e]	23.4[e]	1.07
C18:0	18.0[d]	5.8[e]	8.3[e]	1.52
C18:1 trans	3.2[d]	0.9[e]	1.4[e]	0.41
C18:1 cis	13.7[d]	4.3[e]	3.7[e]	0.28
cis,trans	0.4	0.3	0.3	0.12
C18:2	27.1[d]	7.2[e]	7.4[e]	1.25
C18:2 c-9, t-11	0.3	0.1	0.1	0.13
C18:2 t-10, c-12	0.1	0.0	0.0	0.03
AA	0.0[d]	0.6[e]	0.6[e]	0.10
EPA	0.0[d]	1.3[e]	1.5[e]	0.11
DPA	0.0[d]	9.8[e]	9.7[e]	0.79
DHA	0.0[d]	25.9[e]	24.3[e]	2.19
Others	2.3[a]	3.9[b]	4.4[b]	0.56

B.	2hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	16.5[d]	25.0[e]	24.5[e]	1.52
C18:0	15.3[d]	5.7[e]	5.5[e]	0.45
C18:1 trans	3.8[d]	1.7[e]	1.8[e]	0.22
C18:1 cis	12.4[d]	3.9[e]	3.7[e]	0.40
cis,trans	0.4	0.3	0.3	0.07
C18:2	24.7[d]	5.8[e]	7.0[e]	1.37
C18:2 c-9, t-11	1.0[d]	0.3[e]	0.3[e]	0.04
C18:2 t-10, c-12	0.2[d]	0.0[e]	0.0[e]	0.01
AA	0.0[d]	0.4[e]	0.5[e]	0.05
EPA	0.0[a]	1.0[b]	1.4[c]	0.11
DPA	0.0[d]	7.5[e]	8.5[e]	1.32
DHA	0.0[d]	19.4[e]	20.9[e]	3.14
Others	2.4[a]	3.9[b]	4.0[b]	0.30

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 6. (continued).

C. 4hr

Fatty Acid	Control I	Extract (E)	Intact (I)	SE
C16:0	16.8^{d}	24.7^{e}	25.6^{e}	0.64
C18:0	16.1^{d}	5.9^{e}	5.2^{e}	1.30
C18:1 trans	4.9^{d}	2.4^{e}	2.3^{e}	0.49
C18:1 cis	12.0^{a}	4.3^{b}	3.1^{c}	0.28
cis,trans	0.3	0.4	0.4	0.10
C18:2	23.3^{d}	5.9^{e}	4.8^{e}	3.02
C18:2 c-9, t-11	0.7^{d}	0.2^{e}	0.3^{e}	0.04
C18:2 t-10, c-12	0.1^{d}	0.0^{e}	0.0^{e}	0.02
AA	0.0^{a}	0.4^{b}	0.6^{c}	0.05
EPA	0.0^{a}	1.0^{b}	1.3^{c}	0.06
DPA	0.0^{d}	7.8^{e}	7.5^{e}	0.32
DHA	0.0^{d}	20.3^{e}	19.1^{e}	0.88
Others	2.6^{a}	4.2^{b}	3.9^{b}	0.30

D. 6hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	15.7^{d}	25.7^{e}	24.8^{e}	1.74
C18:0	17.2^{d}	5.7^{e}	5.2^{e}	1.14
C18:1 trans	6.9^{d}	2.8^{e}	2.9^{e}	0.63
C18:1 cis	11.6^{d}	4.2^{e}	3.5^{e}	0.54
cis,trans	0.2	0.4	0.4	0.10
C18:2	21.4^{d}	5.3^{e}	5.0^{e}	1.18
C18:2 c-9, t-11	0.5^{d}	0.2^{e}	0.2^{e}	0.05
C18:2 t-10, c-12	0.1^{a}	0.0^{b}	0.0^{b}	0.02
AA	0.0^{d}	0.4^{e}	0.6^{e}	0.09
EPA	0.0^{a}	1.0^{b}	1.3^{c}	0.09
DPA	0.0^{d}	7.9^{e}	9.2^{e}	0.94
DHA	0.0^{d}	20.1^{e}	21.8^{e}	2.35
Others	2.6^{d}	4.0^{e}	4.2^{e}	0.24

^{a,b,c} Means with different superscripts differ $p<0.05$
^{d,e,f} Means with different superscripts differ $p<0.01$

Table 6. (continued).

E.	24hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	15.5[d]	23.9[e]	24.1[e]	1.33
C18:0	22.5[d]	5.5[e]	4.8[e]	1.33
C18:1 trans	7.8[d]	5.7[e]	5.5[e]	0.25
C18:1 cis	6.7[d]	4.2[e]	3.7[e]	0.39
cis,trans	0.3[a]	0.4[b]	0.4[b]	0.03
C18:2	8.3[d]	3.5[e]	3.9[e]	0.90
C18:2 c-9, t-11	0.3	0.2	0.2	0.09
C18:2 t-10, c-12	0.1	0.0	0.0	0.03
AA	0.0[d]	0.4[e]	0.5[f]	0.03
EPA	0.0[d]	0.8[e]	1.0[e]	0.08
DPA	0.0[d]	8.3[e]	7.9[e]	0.63
DHA	0.0[d]	18.0[e]	18.2[e]	1.11
Others	4.3	5.4	4.5	0.56

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 7. Amount (mg) of LCFA for all treatments at 0hr (A), 2hr (B), 4hr (C), 6hr (D), and 24hr (E) in batch cultures.

A. 0hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	3.8[a]	14.7[b]	18.6[b]	2.63
C18:0	3.8	3.4	6.5	0.92
C18:1 trans	0.7[a]	0.5[a]	1.1[b]	0.10
C18:1 cis	3.0	2.7	2.8	0.31
cis,trans	0.1	0.2	0.3	0.10
C18:2	6.2	4.5	5.7	0.72
C18:2 c-9, t-11	0.1	0.1	0.1	0.04
C18:2 t-10, c-12	0.0	0.0	0.0	0.01
AA	0.0[d]	0.3[e]	0.5[e]	0.07
EPA	0.0[d]	0.8[e]	1.2[e]	0.16
DPA	0.0[d]	5.9[e]	7.5[e]	0.99
DHA	0.0[d]	15.5[e]	18.9[e]	2.48
Others	5.1	2.5	3.5	3.54
Total Lipid	34.7	86.7	94.0	

B. 2hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	3.7[d]	17.5[e]	17.6[e]	1.55
C18:0	3.4	4.0	3.9	0.26
C18:1 trans	0.8[a]	1.2[b]	1.3[b]	0.10
C18:1 cis	2.8	2.8	2.7	0.22
cis,trans	0.1	0.2	0.2	0.05
C18:2	5.6	4.1	5.0	0.64
C18:2 c-9, t-11	0.2	0.2	0.2	0.01
C18:2 t-10, c-12	0.0	0.0	0.0	0.01
AA	0.0[d]	0.3[e]	0.4[e]	0.04
EPA	0.0[d]	0.7[e]	1.0[e]	0.11
DPA	0.0[d]	5.3[e]	6.1[e]	1.02
DHA	0.0[d]	13.7[e]	15.0[e]	2.45
Others	0.5[d]	2.8[e]	2.8[e]	0.22
Total Lipid	47.0	78.7	84.3	

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 7. (continued).

C.	4hr			
Fatty Acid	Control	Extract	Intact	SE
C16:0	4.3[d]	17.9[e]	20.2[e]	1.54
C18:0	4.0	4.3	4.1	0.13
C18:1 trans	1.2	1.7	1.8	0.19
C18:1 cis	3.0	3.1	2.4	0.24
cis,trans	0.1[a]	0.3[b]	0.3[b]	0.05
C18:2	6.1	4.2	3.8	1.17
C18:2 c-9, t-11	0.2	0.2	0.2	0.02
C18:2 t-10, c-12	0.0	0.0	0.0	0.01
AA	0.0[d]	0.3[e]	0.5[f]	0.02
EPA	0.0[a]	0.8[b]	1.0[c]	0.09
DPA	0.0[d]	5.6[e]	6.0[e]	0.57
DHA	0.0[d]	14.6[e]	15.2[e]	1.68
Others	0.7[d]	3.1[e]	3.1[e]	0.19
Total Lipid	42.0	88.3	102.7	

D.	6hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	2.7d	18.3[e]	20.2[e]	1.28
C18:0	4.1	4.0	4.2	0.19
C18:1 trans	1.6	2.0	2.3	0.22
C18:1 cis	3.1	2.9	2.8	0.45
cis,trans	0.1a	0.3b	0.4b	0.04
C18:2	5.8	3.8	4.1	0.85
C18:2 c-9, t-11	0.1	0.1	0.2	0.03
C18:2 t-10, c-12	0.0	0.0	0.0	0.01
AA	0.0a	0.3[b]	0.5[c]	0.08
EPA	0.0d	0.7[e]	1.1[f]	0.06
DPA	0.0d	5.5[b]	7.5[c]	0.78
DHA	0.0d	14.1[e]	17.7[e]	1.84
Others	0.5	2.8[b]	3.4[c]	0.09
Total Lipid	47.0	77.3	110.7	

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 7. (continued).

E.	24hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C16:0	5.1[d]	19.3[e]	19.3[e]	1.38
C18:0	7.3[a]	4.4[b]	3.9[b]	0.88
C18:1 trans	2.5[d]	4.6[e]	4.4[e]	0.22
C18:1 cis	2.2[a]	3.4[b]	3.0[b]	0.27
cis,trans	0.1[a]	0.3[b]	0.3[c]	0.01
C18:2	2.6	2.8	3.1	0.59
C18:2 c-9, t-11	0.1	0.1	0.2	0.03
C18:2 t-10, c-12	0.0[a]	0.0[b]	0.0[b]	0.01
AA	0.0[a]	0.3[b]	0.4[c]	0.03
EPA	0.0[d]	0.7[e]	0.8[e]	0.06
DPA	0.0[d]	6.7[e]	6.3[e]	0.58
DHA	0.0[d]	14.5[e]	14.5[e]	0.88
Others	1.4[d]	4.3[e]	3.6[e]	0.36
Total Lipid	76.0	92.7	111.0	

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

CHAPTER 4

MONITORING MICROBIAL SHIFTS IN RESPONSE TO ANTIBIOTICS AND OIL USING T-RFLP ANALYSIS

ABSTRACT

Antibiotics and supplemental fat are fed to high producing cattle to improve feed efficiency. Their metabolic effects are similar and well known, but it is not clear how they alter the microbial populations in the rumen to achieve these effects. The objective of this study was to investigate microbial population changes in response to the antibiotics monensin (M) and bacitracin (B) and supplemental fat in the form of oil (O). Mixed cultures of rumen microbes were incubated in artificial fermentors for a total of 16 d. Each run consisted of 5 fermentors. One served as a control (C) and received alfalfa hay, while the other four received one of the following four treatments; 1) monensin then oil (MO), 2) oil then monensin (OM), 3) bacitracin then oil (BO) and, 4) oil then bacitracin (OB). Each run was replicated three times (n=3). Fermentation variables measured were volatile fatty acids (VFA), total cell number, methane production, culture pH, ammonia (NH_3), and long-chain fatty acids (LCFA). Samples also were taken to analyze the microbial population using terminal restriction fragment length polymorphisms (T-RFLP).

There were no significant changes in the fermentation parameters in C over time. As expected, both M and O reduced acetate ($p<0.01$), increased propionate ($p<0.05$), and decreased methane production ($p<0.05$). Bacitracin did not alter acetate or propionate but reduced methane ($p<0.05$). The sequence of additive supplementation did not alter rumen fermentation, but it did seem to cause differences in the microbial populations. The T-RFLP of C showed an adaptation to the in vitro system before d4 and indicates a decrease in diversity occurring between d10 and d16. There were differences between M and B in diversity and in divisions at the Class level, reflecting the different mode of

action of the two antibiotics. M and O also were different from each other, suggesting the additives affect different microorganisms. Furthermore, the sequences in which the additives were supplemented affected the microbial populations.

INTRODUCTION

Cows in peak lactation are fed supplemental fat and ionophores to help meet their tremendous metabolic requirements. Individually, these additives increase feed efficiency. Fat or oil (O) provides a more energy dense diet without requiring a fiber reduction (Palmquist and Jenkins, 1980), and ionophores improve the feed:gain ratio (Pressman, 1976) by altering rumen fermentation and the pattern of volatile fatty acids. In the United States, monensin (M) has been fed to beef cattle since 1975, to lactating dairy cattle since 2005, and is the most commonly tested and used ruminant ionophore.

Both O and M improve the fermentation profile, as they decrease acetate:propionate and reduce methane production (Jenkins, 1993, Russell and Strobel, 1989). The effects of O and M on pH are inconsistent (Jenkins, et al., 2003). Because of their ability to alter the fermentation of the rumen, O and M likely have effects on the microbial population (Jenkins, et al., 2003, Sauer, et al., 1998, Van Nevel and Demeyer, 1995). Supplemental fat may physically coat fiber, bind to microbial cells and affect the cell membranes, and may change competition, and thus the populations, in the rumen (Palmquist and Jenkins, 1980). Monensin is most active against Gram (+) bacteria, which lack much of the protective outer membrane that makes Gram (-) bacteria less susceptible to the antibiotic (Russell, 1987). Monensin is an antiporter that disrupts the ion gradients in the microbial cell, depleting the ATP supply, and inhibiting cellular growth (Russell, 1987).

Bacitracin (B), a less commonly fed antibiotic, is a cyclic peptide that also can be fed as a growth promoter (Butaye, et al., 2003) and produces similar effects as M (Russell and Strobel, 1988). It also acts on Gram (+) bacteria (Russell and Strobel, 1988), but

unlike M, which disrupts the cell membrane, B inhibits cell membrane synthesis (Siewert and Strominger, 1967). Bacitracin has fermentation effects similar to M, but its effects are during growth of the cell (Russell and Strobel, 1988).

Although M and O have beneficial affects on fermentation when fed alone, *in vivo* studies have shown that when fed together, they do not improve efficiency (Clary, et al., 1993, Zinn and Borques, 1993). Similar results have been found *in vitro*, as Jenkins et al. (2003) found that when fed together, O and M decreased propionate, increased A:P, and did not change methane.

Although M likely inhibits Gram (+) bacteria, that description may be an oversimplification. Gram (+) bacteria hydrogenate 18:2 to 18:1 and this step can occur during M supplementation (Fellner, et al., 1997, Sauer, et al., 1998). Lipolytic and hydrogenating bacteria are reported to be mostly Gram (-) and given that M has been shown to inhibit lipolysis, it is hypothesized that M may change the metabolism of Gram (-) bacteria as well as inhibit Gram (+) bacteria (Van Nevel and Demeyer, 1995).

The objectives of this study were 1) to determine the effects of antibiotics and oil on rumen fermentation and 2) to monitor the effect of the additives and the sequence of the additives on rumen microbial populations. It was hypothesized that although the changes in fermentation by M, O, and B are similar, they have different effects on the microbial population of the rumen due to their different mode of actions. Because of their different modes of action, the sequence in which the additives are supplemented would have different effects on the populations in the rumen.

Because of limitations with culture-based techniques, molecular methods are necessary to better characterize the populations in the rumen and in other microbial

environments. Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu, et al., 1997) has been used to study communities of microorganisms and has been shown to be a specific, reliable, and reproducible method for studying such populations (Dunbar, et al., 2001, Osborn, et al., 2000). Although becoming an established method in microbiology, it is not commonly used to profile populations in the rumen.

MATERIALS AND METHODS

Rumen fluid collection and fermentor set-up

Rumen fluid was collected from a ruminally fistulated non-lactating Holstein cow consuming a predominantly forage diet and housed at the North Carolina State University Dairy Educational Unit. The care and use of the animal was approved by the North Carolina State University Institution of Animal Care and Use Committee. Approximately six liters of fluid were sampled from various sections in the rumen using a manual hand held pump and, along with a handful of rumen digesta, placed in a sealed, preheated insulated container, and transported to the laboratory. Once in the lab, the fluid was filtered through double-layered cheesecloth, mixed thoroughly, and 700 mL were added to each of five dual-flow fermentors.

Several hours before the addition of ruminal inoculum, the fermentors were purged with CO_2 to displace any oxygen, heated to 39°C, and the saliva line was primed. Throughout the trial, the water bath maintained the fermentor temperature at 39°C, CO_2 flow was set at a constant rate at 20.0 ml/min, and artificial saliva was delivered with a precision pump at 0.73mL/min that resulted in a fractional liquid dilution rate of 6.8%/hr. The fermentors were continuously mixed at 10 rpm.

Experimental Protocol

Diet mixing

Alfalfa hay used in this experiment consisted of pellets and was purchased from a local feed store. M and B were purchased from Sigma Chemical Co. (St. Louis, MO), and soybean oil (O) (Wesson brand) was purchased at a local grocery store. At time of mixing, one kilogram of alfalfa hay per diet was added to a blender and 22mg of the respective antibiotics were added to yield a concentration of 22ppm. This level was chosen because it is the highest level approved for inclusion in lactating dairy cattle diets. The diets were thoroughly mixed to assure complete distribution of the antibiotics. The feed was deposited in the liquid portion of the contents. Supplemental oil (375µl) was added directly to the fermentors at each feeding via a long Pasteur pipette.

Dietary Treatments

The trial schedule is shown in Table 1. A 100% forage diet consisting of alfalfa pellets was fed in equal portions at 0700 and 1400h, for a total of 13.5 g of feed (DM basis) per day. The fermentors were allowed a 4 d stabilization period, during which time all fermentors received a 100% alfalfa pellet diet. On d5, two fermentors received O at 5% of diet DM, one fermentor received M at 22ppm of diet DM, and one received B at 22ppm. These dietary treatments were monitored for 6 d. On d11, one of the two fermentors that received 5% O also received M (22ppm) and the other fermentor received B (22ppm). The other two fermentors that received only M or B then received 5% O. These diets were allowed to ferment for an additional 6 d. Each run consisted of a total of 6 fermentors. One fermentor was used as a positive control (C) and received 100% alfalfa pellets throughout the experiment. Each run was replicated 3 times.

Sample collection and analytical procedures

Sample Collection

Samples for cell counts were collected on d4, 5, 9, 10, 11, 15, and 16. Samples for VFA, LCFA, ammonia, and T-RFLP were obtained two hours after feeding on d4 (the last day of adaptation) and d10 and d16 (the end of each dietary treatment). Methane and pH were measured daily. Prior to sampling culture contents, the speed on the automatic stirrers was increased to thoroughly mix fermentor contents. A pipette with a large opening was used to obtain a homogenous sample.

Methane and pH

Methane and pH were recorded everyday of the trial. Methane gas measurements were obtained by filling a gas-tight syringe (Hamilton Co., Reno, NV) with 10µl of fermentor headspace gas and directly analyzed using gas chromatography (model CP-3380; Varian; Walnut Creek, CA) according to Fellner, et al. (1997). Methane measurements were made at -1, 1, 2, 3.5, 5, and 6.5h after feeding. The pH was continuously monitored by a pH probe (Cole Parmer; Vernon Hills, Illinois) suspended in the rumen fluid and recorded at the time of methane analysis. Hourly methane and pH values were averaged across days. Daily means of the last two days of each treatment period were used for statistical analysis.

VFA and Ammonia

On d4, 10, and 16 at 0900h, two 1ml samples were obtained for VFA and ammonia analysis. The samples for ammonia were immediately stored at -70°C. The VFA samples were immediately processed by suspending in metaphosphoric acid and centrifuging in a tabletop centrifuge (model Micromax; International Equipment Co.;

Needham Heights, MA) at 21,000g for 10 minutes. The samples were then stored at -70°C until subsequent analysis. Ammonia was analyzed by a colorometric procedure (Beecher and Whitten, 1970) and the VFA were analyzed by gas liquid chromatography (model CP-3380; Varian; Walnut Creek, CA) using a fused silica capillary column with 30m x 0.25mm x 0.25µm film thickness (Nukol; Supelco Inc., Bellefonte, PA) according to Fellner, et al. (1997).

Fatty Acids

On d4, 10, and 16, 5mL samples were taken for LCFA analysis two hours after the morning feeding. Fatty acids were extracted using the Bligh and Dyer method (1959), methylated by the procedure outlined by Kramer (1997) and analyzed by gas liquid chromatography (model CP-3380; Varian; Walnut Creek, CA). The column used was a fused silica capillary column (Nukol; Supelco Inc., Bellefonte, PA) with 100m x 0.25mm x 0.2µm film thickness. The injector temperature was 230°C. The oven temperature was held at 70°C for 4 minutes and then increased 13°C per minute until reaching 175°C, where it remained for 27 minutes. It then increased 8°C per minute to 215°C, where it remained for 45 minutes, for a total time of 89 minutes. The flame ionized detector (FID) temperature was 250°C.

Cell counts

Cell counts were obtained on the fresh inoculum and on culture contents 2h after feeding on d4, 9, 10, 15, and 16. Additional samples for cell counts were taken 5h after feeding on d5 and 11. Cell counts were done with a phase-contrast microscope (Nikon Type 119; Japan) using a hemocytometer (Hausser Scientific; Horsham, PA) after

diluting the sample with LB broth. Cell counts from d4, 10, and 16 were averaged across replicates and statistically analyzed.

T-RFLP analysis

A 10 mL sample was taken from the inoculum and on d4, 10, and 16, 10 mL samples were taken from each fermentor two hours after the morning feeding and stored at -70°C until analysis for T-RFLP (Liu, et al., 1997). Samples were thawed and DNA was extracted with the PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc.; Solana Beach, CA). A 1µl sample of each genomic DNA (gDNA) was run on a 1% agarose gel at 120 volts for 60 minutes. Bacterial and archaeal specific PCR reactions were performed in duplicate on 1µl of the purified gDNA using the bacterial primers 8F (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and the archaeal primers Ar109F (5'-AC(G/T)GCTCAGTAACACGT-3') and Ar912R (5'-CTCCCCCGCCAATTCCTTTA-3'). Each PCR reaction consisted of 100µl, containing 10µl 10X buffer, 0.8µl dNTP, 0.5µl fluorescently labeled forward primer, 0.5µl reverse primer, 0.5µl taq polymerase, 86.7µl PCR grade water, and 1µl gDNA. The PCR was performed on an iCycler® (Bio-Rad Laboratories; Hercules, CA) and the program began with 3 minutes of denaturing at 94^{o}C, followed by 25 cycles of 94^{o}C at 1 minute, 50^{o}C at 1 minute, and 72^{o}C at 2 minutes, and finished with 7 minutes of extension at 72^{o}C. One µl of each PCR reaction was run on a 1% agarose gel at 120 volts for 60 minutes.

The two PCR reactions of each sample were pooled and purified using the UltraClean™ PCR Clean-up Kit (MoBio Laboratories, Inc.; Solana Beach, CA) and 1µl of each purified PCR reaction was run on a 1% agarose gel at 120 volts for 60 minutes.

The remaining 49µl of the PCR products were then digested in three separate reactions. Each of three tubes contained 15µl of the purified PCR reaction, 74µl of PCR grade water, 1µl of one of three enzymes: *Rsa*I, *Msp*I, or *Hha*I, and 10µl of its corresponding buffer. The *Hha*I reaction required 1µl of BSA and contained 73µl of water. All enzymes were purchased from New England BioLabs (http://www.neb.com). The digestion reactions were incubated overnight at 37 ^{o}C and heat killed by immersing in a 60-70 ^{o}C water bath for 20 minutes. The reactions were purified using the QIAquick Nucleotide Removal Kit (Qiagen, Inc.; Valencia, CA) and eluted with 50µl of heated PCR grade water. Samples of 20µl from the purifications were run on a 1% agarose gel at 120 volts for 60 minutes. The remaining 30µl of the samples was wrapped in parafilm and stored at -80°C until shipped to the Genomic Technology Support Facility at Michigan State University for analysis on an Applied Biosystems Prism 3100 Gene Analyzer (Applied Biosystems; Foster City, CA). The resulting data files were uploaded and filtered so only information associated with the added fluorescent tag remained. The resulting fragment patterns were analyzed using InSilico, Inc (http://www.insilicoinc.com/).

Statistical analysis

All fermentation data and total cell numbers were analyzed using the Proc Mixed procedure of SAS (SAS Institute; Cary, NC). The random variable was replicate and the significance is reported at $p<0.05$.

RESULTS

Description of data generated from T-RFLP analysis

Fragment Utilization

The total unique fragments were enumerated using the T-RFLP generated by digestion with *Rsa*I, *Hha*I, and *Msp*I. Every combination that included one fragment from each digestion created a unique fragment pattern, which may correspond to a unique species. The fragment pattern does not reveal the organism number per species, but does provide information on the diversity of the population. The percentages of fragments not utilized by the InSilico database (unmatched fragments) are presented in Table 2. Discarding the highest and lowest percentages for each enzyme resulted in averages of 84%, 92%, and 90% for the *Rsa*I, *Hha*I, and *Msp*I generated T-RFLP, respectively. The highest utilization of fragments was 100% of the H*haI* digested BO sample, followed by 98% of the M*spI* digested Cd4 and B samples, and 95% of the R*saI* digested O sample. The lowest utilization of the *Hha*I generated fragments was from the M sample (79%) and the lowest from the *Msp*I fragments was from the MO treatment (69%). The *Rsa*I digested fragments that had the lowest utilization were from the MO treatment, which only had 46% T-RF utilization. This was the lowest percentage for all enzymes across all treatments. However, Table 5 shows that there were also more T-RF (29) generated from R*saI* digestion of the MO sample than any enzyme generated for any sample for any replicate. Table 5 shows the comparisons of the T-RFLP generated for all three replicates using the bin tolerances according to Kent, et al. (2003). Table 6 shows the comparisons among replicate 1, and Table 7 shows the comparisons of T-RFLP generated by the archaeal primers in replicate 1.

Description of Classifications

Unclassified fragments are different from unmatched fragments. They are matched to restriction sites in the database, but because little or no phylogenetic information is deposited with their sequences, it is not possible to determine the identity of the species belonging to the fragment patterns. Unclassified sequences make of up a large percentage of the data, as sequences are often deposited without being identified. However, the sequences are often named based on where the sample was collected, such as uncultured soil bacterium clone, swine fecal bacterium, or uncultured freshwater bacterium clone.

Fragment patterns in the Multiple division are like the Unclassified fragments in that their identity cannot be determined with certainty. However, they differ in that the fragment patterns match more than one species and it is not possible to tell which species are actually present in the sample. The Multiple category consists of all the unique species that could match a particular fragment pattern. There may actually only be one species per fragment pattern present in the sample, or a pattern could be generated from the presence of several unique species in the sample. For each fragment pattern in the Multiple division, InSilico generates a list of the possible species that have the particular fragment pattern. To definitively classify the species in the Multiple classification, further phylogenetic work should be done on the sequences in the database or on future sequences before they are deposited. Also, the Multiple classification includes different species with the same fragment patterns, which means they have the same restriction sites for *Rsa*I, *Hha*I, and *Msp*I, so a different restriction enzyme combination may be necessary to distinguish between the species producing the similar fragment patterns.

Control Fermentors

Variation in fermentation parameters and cell numbers over 16d

The effect of time on the control fermentors is shown in Figures 1B, 2, and 3. There were no significant changes in the fermentation profile of the control fermentors over time. Methane production averaged 28 mmol/day, pH was 6.0, and ammonia (data not shown) averaged 24.3 mg/100mL over the 16d period. The daily pH values, methane production (Figure 2), and ammonia production were unaffected by time. Total VFA was numerically greater on d16, but averaged 55.7 mM over the experiment and was not significantly different from d4 or d10. Neither total nor individual (data not shown) VFA mM concentrations differed, and the A:P remained constant at 3.8 throughout the trial (Figure 3). Total cell counts are shown in Figure 1. The total cell number for the inoculant was always higher than those for the culture contents (data not shown). The cell numbers in the control fermentors averaged 1.35 E9 and did not change significantly over time (Figure 1B).

Variations in bacterial populations

The number of unique fragment patterns sorted by Class for all treatments is shown in Figure 4. The total number of unique species identified in the control fermentors was similar between d4 and d10, but appeared to decrease by d16. The unique fragment patterns were 281, 264, and 125 at d4, d10, and d16, respectively. Unclassified unique fragment patterns followed the same trend, as there were 144 unclassified patterns on d4, 120 on d10, and 58 on d16. Similarly, the Multiple classification remained very similar between d4 and d10 (72 and 73) and was halved to 35 by d16.

There was a shift in the number of unique species in *Actinobacteridae*, *Bacteroidetes*, and *Gammaproteobacteria* (Figure 4). The smallest change between days was a difference of 1 unique fragment pattern and the largest was 4 unique patterns. More of a shift occurred in the *Actinobacteria*, *Betaproteobacteria*, and *Epsilonproteobacteria* classes with the smallest variation being one fragment pattern and the largest seven. Across time, the unique species assigned to a Class did not differ more than 2 species for the *Mollicutes* and *Sphingobacteria* classes and no more than one species in the remaining classes, if any at all. There were decreases in the *Bacilli* and *Deltaproteobacteria* classes across time. Overall, the unique fragment patterns sorted at the Class level for the control fermentors were consistent between d4 and d10 and seemed to decrease by d16.

The fragment patterns were calculated on a percent basis and shown in Figure 5. The proportion of Unclassified fragment patterns ranged from 45.5% to 51.2% and the proportion of unique fragments in the Multiple Class ranged from 25.6% to 28%. Although the number of fragment lengths in both of these Classes differed, the proportions of each, as suggested by Class level information, remained very similar over time. In contrast, however, *Gammaproteobacteria* numbers of fragments were very close, but there was an increase in its proportion over time, from 4.4 to 6.1% to 11.2% on d4, d10, and d16, respectively.

The unique fragment patterns per genus are presented on a percentage basis in Figure 8A. The graph shows the breakdown of unique species of eleven important rumen genera. The Identified species are unique fragments assigned to only one genus, and the Multiple category includes fragment patterns that may belong to more than one genus.

The Multiple categories were searched for genera of interest with the knowledge that the presence of the genus in the sample cannot be confirmed without digestion by another enzyme. Figure 8 includes both the identified genus and the possible genus presence (Multiple) in the treatments. The breakdown of species by genus may help explain the cause of the shifts in classes.

Because deposited sequences are often named based on where the sample was collected, the Multiple and the Unclassified categories were searched for sequences collected from the rumen. Although phylogenetic information is not known about these sequences, it is possible to determine which fragment patterns could belong to microorganisms sampled from the rumen. Unclassified sequences make of up a large percentage of the data, so searching the categories ensures that not as much data are lost in the larger divisions. Figure 9 shows the breakdown of the Multiple and Unclassified divisions after being searched for the word rumen in their name. The keyword rumen appeared in the Unclassified division only as uncultured or unidentified rumen bacterium, but it was in the Multiple category with other bacteria that have more specific identification.

Variations in archaeal populations

Figure 10 shows the areas of the fragment lengths generated by amplifying the 16S rDNA of the C and treatment samples with an archaeal primer followed by digestion with *Rsa*I, *Hha*I, and *Msp*I. Although the T-RFLP from one digestion cannot determine the identity of the microorgansims (Marsh, 1999), possible shifts in the archaeal population may be inferred. The figures show the appearance and disappearance of various terminal fragments over time and illustrate the archaeal populations in continuous

culture. The presence and absence of fragments have been used to interpret T-RFLP data (Thies, 2007). The 160, 184, and 795 bp fragments resulting from *Rsa*I digestion are present in all C samples, at d4, d10, and d16, as shown in Figure 10A. The fragments created by *Hha*I digestion are shown in Figure 10B. There are no fragments common to all three sample days. Figure 10C shows the areas of the fragments generated by *Msp*I digestion and show that several fragments are present in the C across time, fragments 62, 107, 203, 206, 437, and 443 bp in length. All three Figures show appearance and disappearance of various fragments with the biggest difference occurring at d16.

Effect of Additives

Effect of additives on microbial fermentation and cell counts

The effects of the additives on total and individual VFA concentration is shown in Table 3. Total VFA (mM) production was not significantly different between additive treatments, and averaged 49.8 mM for C and 49.2 mM, 52.8 mM, and 48.6 mM for the fermentors treated with M, B, and O, respectively. There was an additive (x) sequence interaction for total VFA production. The addition of B or O alone did not affect total VFA, but the sequence in which they were added resulted in significantly higher total VFA mM ($p<0.05$) in BO than in B.

Millimolar concentrations of acetate (A), propionate (P), and isobutyrate also did not differ between additive treatments. However, M, B, and O treatments resulted in significantly higher butyrate ($p<0.05$) than C. Isovalerate and valerate were not affected by M and B, but were greater with O compared to C.

As expected, both M and O decreased the molar percentages of acetate ($p<0.01$) and increased that of propionate ($p<0.05$) compared to C, which also decreased the A:P

ratio ($p<0.05$). The molar percentages of acetate and propionate were not significantly altered by B when compared to the untreated fermentors. All additives (M, B, and O) caused an increase in butyrate ($p<0.01$) and an increase in valerate ($p<0.01$) when compared to C. M and O increased the molar proportion of isovalerate ($p<0.05$), while B had no effect when compared to C.

The affects of additives on methane, culture pH, ammonia concentration, and digestibility are shown in Table 4. All three additives significantly decreased methane production ($p<0.05$). The decrease in methane was greatest with M and O (40.5% and 35.5%, respectively), with the decrease by B being lower (17.6%). Both M and B increased pH to 6.2 and 6.1, respectively from the 6.0 of C ($p<0.01$) and O decreased it ($p<0.01$) to 5.9. None of the additives had a statistically significant affect on cell numbers or ammonia concentration.

Effect of additives on bacterial populations

The total number of unique fragment patterns sorted by Class for all additives and their sequence is shown in Figure 4. Both M and O seemed to decrease the total number of unique fragment patterns when compared to C, as the additives dropped them from 281 to 177 and 234, respectively. Treatment with B, however, increased the total by more than twice as many to 612. The same pattern occurred with the number of unique species in both the Unclassified and Multiple divisions, as M nearly halved the number of unique fragment patterns in the Unclassified division when compared to control (144 to 82), B nearly doubled them (to 272), and O decreased them, but not as dramatically as M (to 119). M decreased the number of species classified in the Multiple division from 72 to 42, B more than doubled them to 153, and O decreased them to 52.

M treatment resulted in one unique species in both the *Cloroflexi* and *Deferribacteres* classes that was not present in the C fermentors and did not appear in either B or O treatment. It also resulted in no unique fragment patterns in either *Flavobacteria* or *Nitrospira* although unique species in each Class were observed in C as well as during B and O treatment. M reduced the unique species in *Epsilonproteobacteria* while B and O had no affect.

The number of unique species classified in *Actinobacteria* were 10 in the C and remained unchanged at 9 in O. M, however, halved the species to 4 and B doubled them to 21. There were 11 different species in *Deltaproteobacteria* in C and this was similar to 10 in M and 11 in B, however, O reduced it to 3 unique species, which is one forth of that observed in all other treatments.

The C fermentor had one unique species classified in *Acidobacteria* as did B. No species of this Class were present after M or O treatment.

When compared to C, B increased the number of unique fragment patterns in several classes, including *Actinobacteridae*, *Alphaproteobacteria*, *Bacilli*, and *Clostridia*, while neither M nor O altered the number of unique species considerably. The unique species in *Verrucomicrobiae* were similar in C and B, but were not present in M or O. However, B treatment resulted in four unique species in *Mollicutes* that were not present in C and that did not also appear in M or O. The number of unique species classified in *Sphingobacteria* increased from 1 to 2 in B, but did not change between the C, M, or O treatments.

Compared with C, M did not affect the species in beta- and *Gammaproteobacteria*, while B and O increased them. *Gammaproteobacteria* had 13

unique fragment patterns in C, 14 in M, 20 in O, and 74 unique species in B. Unique species of *Betaproteobacteria* in C was 6, in M was 8, in O was 10, and in B was quadruple the number of C at 27. The three additives did not alter the number of unique species by more than one in the *Bacteroidetes* class, and there were not any species in the *Chlorobia* Class for the C or treated fermentors.

When the number of unique species per Class are calculated as a percentage of total unique fragment patterns, the Class distribution is more similar across treatments. The proportion of unique species found in the Unclassified division is lowest in B at 44.4%, more in M at 46.3% and highest in O at 50.9%, O being very close to the 51.2% in C. The percentage of fragments in the Multiple classification followed the reverse trend, as it was lowest in O at 22.2%, more in M at 23.7%, and greatest in B at 25.0%. B most closely matched the proportion of 25.6% observed in C. The percentage of *Gammaproteobacteria* increased with all treatments, as M and O almost doubled it to 7.9% and 8.5% and B almost tripled it to 12.1%.

The greatest change in unique species as sorted by Class occurred with B. However, the greatest percentage change in species was with M. M decreased the proportion of *Actinobacteria* from 3.6% to 2.3%, while B and O only changed it by 0.2% to 3.4 and 3.8%, respectively. M increased the proportion of *Bacilli* from 2.7% in C to 4.0%, while B and O only changed it to 2.1% and 2.8%, respectively. M increased *Bacteroides* from 0.5% to 1.1%, which followed the C at d10, while B and O were very similar to C at d4. M also increased the proportion of species classified in *Deltaproteobacteria* from 3.9% in C to 5.6%, while B and O lowered it to 1.8% and 1.3%, lower than in C at d10.

All three treatments increased *Actinobacteridae* by about 1-2% when compared to C and they all more than doubled the proportion of *Betaproteobacteria* from 2.1% in C to 4.3-4.5%.

Effect of additives on archaeal populations

The effect of additives on the archaeal fragment areas are presented in Figure 11. The *Rsa*I digestion generated several fragment lengths, fragments 160, 184, and 531 bp, that were present across all treatments. Five of the *Hha*I generated fragments, 112, 113, 246, 256, and 260 bp, were present in all treatments. However, several fragments were only detected in the B and O treatments. *Msp*I digestion also generated several fragments that were common to all treatments and those were 62, 107, 206, 437, and 443 bp in length.

Effect of Sequence

Effect of sequence of additives on microbial fermentation and cell counts

The sequence in which the additives were included had no major effect on microbial fermentation patterns (Tables 4-5). There was an additive (x) sequence interaction for total VFA production. The addition of B or O alone did not affect total VFA, but the sequence in which they were added resulted in significantly higher total VFA mM ($p<0.05$) in BO than in B. The cell numbers, pH values, methane production, and ammonia production were unaffected by sequence. Neither total nor individual VFA was significantly different between sequences and the A:P remained constant across sequences. There was, however, a sequence effect of M and O, as adding M first (MO) resulted in a pH of 6.0 and adding O first (OM) produced a pH of 5.7.

Effect of sequence of M and O on bacterial populations

The number of unique fragment patterns sorted by Class for all additive sequences is shown in Figure 4. MO had a lower total number of unique fragment patterns when compared to OM (88 vs. 216), and less than half the number of unique patterns in both the Unclassified and Multiple divisions as OM, with 44 vs. 113 and 20 vs. 54, respectively. MO treatment resulted in no species in the *Gammaproteobacteria* class, while OM had 14 unique fragment patterns in the class. Treatment with MO resulted in twice as many unique species identified in the *Bacilli* (8), *Clostridia* (5), and *Alphaproteobacteria* (2) classes than OM treatment, as OM had 4 unique species in *Bacilli*, 2 in *Clostridia*, and 1 in *Alphaproteobacteria*. Fermentors receiving MO treatment had one unique species in both the *Acidobacteria* and *Actinobacteridae* classes that OM did not have. Adding M to fermentors treated with O (OM) yielded twice the number of unique fragment patterns in *Betaproteobacteria* (3 vs. 6) and *Epsilonproteobacteria* (2 vs. 1) than treating with O after M (MO). The OM fermentors also had unique species in classes that were not present in MO, as they had fragment patterns matching one species in both *Bacteroidetes* and *Chloroflexi* and two unique species in *Sphingobacteria*. OM had more species in *Actinobacteria* and *Deltaproteobacteria*, as it had 6 and 9 respectively and MO had only one unique species in each class. Both MO and OM had one unique fragment pattern in *Deferribacteres*. Neither MO or OM had fragment patterns matching species in *Chlorobia*, *Flavobacteria*, *Mollicutes*, *Nitrospira*, *Planctomycetacia*, or *Verrucomicrobia*.

When analyzed on a percentage basis, OM had a greater proportion of both Unclassified and Multiple species, at 52.3% and 25%, compared to 50% and 22.7% in

MO. While MO had no species in *Gammaproteobacteria*, the species in that Class accounts for 6.5% of the unique fragment patterns in OM.

For the classes whose unique species differed two fold, the percentage each contributed to total species, ranged from 0.2% to 7.2%. The largest difference among these classes between MO and OM were in *Bacilli* (9.1% vs. 1.9%), *Clostridia* (5.7% and 0.9%), and *Epsilonproteobacteria* (2.3% and 0.5%). *Betaproteobacteria* proportions were 3.4% for MO and 4.8% for OM, although the number of species classified in each differed two fold.

The proportions of classes between MO and OM for which one of the treatments had at least one unique species while the other had zero did not differ more than 1.5%. The proportion of *Actinobacteria* differed between MO (1.1%) and OM (2.8%). *Deltaproteobacteria* differed between MO (1.1%) and OM (4.2%). The proportions of two classes did not differ more than 1% between MO and OM, *Deferribacteres* and *Alphaproteobacteria*.

Effect of sequence of M and O on archaeal populations

The effect of sequence on the archaeal fragment areas are presented in Figure 12. *Rsa*I digestion of MO and OM treated samples resulted in fragments at 50, 160, 183, 188, 531, and 797 bp. Upon *Hha*I digestion, fragments in both MO and OM were 112, 113, 245, 256, and 260 bp long. There seemed to be the most dramatic effect of sequence between 150 and 228 bp and above 261 bp. The fragments generated in both MO and OM from *Msp*I digestion were 62, 107, 209, and 437 bp.

Effect of sequence of B and O on bacterial populations

BO treated fermentors had fewer total unique species than OB as well as fewer patterns in both the Unclassified and Multiple divisions. Total unique fragment patterns in BO were 197, while OB yielded 346. There were 94 and 168 Unclassified unique species and 49 and 79 belonging to the Multiple division from BO and OB, respectively.

The addition of O after treatment with B (BO) resulted in increased unique fragments in the *Actinobacteria* (10 vs. 8) and *Epsilonproteobacteria* (8 vs 6) classes. There were unique fragment patterns in the *Acidobacteria* (1) *Actinobacteridae* (3), *Chlorobia* (1), and *Flavobacteria* (2) classes in BO but none in those four classes in OB. Altering the sequence to OB resulted in more unique species identified than BO in the classes *Bacilli* (12 vs. 2), *Betaproteobacteria* (12 vs. 9), *Clostridia* (8 vs. 1), *Deltaproteobacteria* (7 vs 2), *Mollicutes* (9 vs. 1), and *Gammaproteobacteria* (32 vs. 12). OB treatment yielded one unique species in each of the *Alphaproteobacteria*, *Deferribacteres*, and *Planctomycetacia* classes while there were no species classified in those divisions for BO.

Both BO and OB had one unique species in *Bacteroidetes* and *Sphingobacteria*. Neither BO or OB had unique species in the Cloroflexi, *Nitrospira*, or *Verrucomicrobiae* classes.

On a percentage basis, the difference between BO and OB in Unclassified species differed by less than 1% (48 vs. 48.6%), while the difference between BO and OB in species in the Multiple division was 25% and 22.8%. Four classes had proportions that differed more than 2% between BO and OB. The two classes that were greater in BO were *Actinobacteria* (5% vs. 2.3%) and *Epsilonproteobacteria* (4.0% and 1.7%), while

the two classes that were proportionally greater in OB were *Bacilli* (1.0% vs. 3.5%) and *Gammaproteobacteria* (6% and 9.2%).

Three classes differed less than 2% between BO and OB: *Betaproteobacteria* (5 vs. 3.5%), *Clostridia* (1 vs. 2.3%), and *Mollicutes* (1 vs. 2.6%). Among the classes that did not have species represented in one of the treatments, the most difference between BO and OB was 2% in the *Actinobacteridae* Class and the others (*Acidobacteria*, *Alphaproteobacteria*, *Chlorobia*, *Deferribacteres*, *Flavobacteria*, *Planctomycetacia*) differed 1% or less. The difference between the proportion of the remaining four classes was 1% or less.

Effect of sequence of B and O on archaeal populations

The effect of sequence on the archaeal fragment areas are presented in Figure 12. When given +/- 1 base pair tolerance (Kent, et al., 2003), *Rsa*I digestion of BO and OB treated samples resulted in fragments at 160, 183, 188, and 531 bp. Upon *Hha*I digestion, fragments in both BO and OB were 112, 113, 246, 256, and 260 bp long. There seemed to be the most dramatic effect of sequence at 104 bp and below, between 150 and 228 bp, and above 261 bp. The fragments generated in both BO and OB from *Msp*I digestion were 62, 107, 203, 206, 210, 444, and 437 bp.

DISCUSSION

Control Fermentors

As expected, the fermentation was very stable in the control fermentors over time (Rufener, et al., 1963). There were no significant changes in methane production, pH, or ammonia concentration. Also unaffected were total and individual VFA as well as A:P.

Cell numbers of the inoculum were greater than the cell numbers of the samples from the fermentor (Ziemer, et al. 2000). The cell numbers in the control fermentors did not change significantly over time. One objective of this study was to monitor any changes in microbial populations in control fermentors in order to better characterize shifts in treatment ferementors. The number of unique species in the controls seemed to drop between d10 and d16, which could indicate a change in diversity over time. Because the diversity seemed to change, but the total cell number did not, changes in microbial populations must be due to changes in the number of unique species rather than changes in the number of organisms per species. The total unique species as well as the species in the Multiple and Unclassified divisions indicate a change in diversity over time. The biggest change occurred between d10 and d16, suggesting that adaptation to the fermentors had occurred before d4 and that changes in diversity due to the continuous culture environment did not happen until after d10. Coupled with the cell counts, this shows that the decrease in diversity over time in the control fermentors is because of a decrease in unique species and not in organism number. This also shows that the cultures do not cause the loss of microorganisms over time. It could mean that specific species of organisms become more acclimated to the in vitro environment over time. Also, because this study was not replicated *in vivo*, it cannot be ruled out that changes in diversity over time are not also a natural occurrence in the rumen.

Based on the changing fragment lengths of the control fermentors, there was apparently a natural shifting of archaeal populations over time in continuous culture. However, as with the bacterial population data, the similarities between Cd4 and Cd10 indicate that adaptation to the *in vitro* system occurred by if not before the fourth day,

and that any shifting in the population did not occur until after the tenth day. Also, because methane production did not change over time in the control fermentors, but it appears there were fluctuations in the archaeal population, there seem to be several methanogens active in producing methane in the rumen.

As a whole, the fermentation and microbial populations of the control fermentors appeared to be stable over time.

Additive Effects

The fermentation characteristics of the treated fermentors were expected, as M decreased A:P, decreased acetate, increased propionate, and decreased methane and O caused similar results (Jenkins, et al., 2003, Russell, 1987). The similar but less drastic effects of B were also expected (Russell and Strobel, 1988). Finally, the lack of significant changes in fermentation due to sequence also was expected. Workers have found that the combination of antibiotics and fat do not seem to have additive effects on fermentation (Jenkins, et al., 2003).

When B affected fermentation, it was similar to M and O, but the T-RFLP data shows B to have many different effects on the microbial population than the M and O additives. This may be explained by the different mode of action of B (Siewert and Strominger, 1967). Therefore, the T-RFLP differences between M and O may also be because the additives have different mode of actions.

Based on information gathered at the Class level, M seemed to reduce diversity while B seemed to increase it. Although M lowered the number of unique species identified, it also increased the number of unmatched fragments. Therefore, M treatment could decrease the number of unique species in the sample, or M could allow for the

growth of bacteria that have not been studied and sequenced. Either effect appears to be occurring without affecting the total number of organisms.

There did not seem to be a large change in the *Bacillus* or *Streptococcus* genera, which is supported by a lack of fluctuation in the *Bacilli* Class with treatment. Because B treatment resulted in the only fragment pattern determined to be in the *Actinomyces* genus across additives, this genus may help to explain the apparent decrease after M and increase after B in the *Actinobacteria* Class. The *Streptomyces* genus, however, shows more identified unique fragment patterns in M and O than in C and B, so other genera within the *Actinobacteria* Class must be responsible for the changes induced by M and B treatment.

The unique species in the Pseudomonas and Vibrio genera seemed to be increased upon B treatment and Pseudomonas increased with M, which could help explain some shifts in the *Gammaproteobacteria* Class. Although the two Gram (-) genera did not appear to fluctuate enough to fully explain the increase of unique species in the Class upon M, B, and O treatment, they do indicate that the increase in *Gammaproteobacteria* may be due to increases in other Gram (-) organisms such as *Ruminobacter*.

The *Bacteroides* genus could possibly explain the increase of species in *Bacteroidetes* upon M treatment, as species in that genus appear to be increased by M and inhibited by B. O did not seem to alter *Bacteroides* genus or the *Bacteroidetes* Class.

The unique species in the *Eubacteria*, *Selenomonas*, and *Megasphaera* genera do not seem to adequately explain the apparent changes in the *Clostridia* Class with treatment. Therefore, other members of *Clostridia* such as Butyrivibrio and Lachnospira may be causing changes. The Class contains Gram (+), Gram (-), and Gram variable

bacteria, so fluctuations in one were expected to be balanced by changes in the other. The *Ruminococcus* genus correlated with the changes in the *Clostridia* and may help explain them.

The fragment lengths that were produced by *Rsa*I digestion that were the same in C over time were also present after additive treatment, suggesting their importance in the rumen and presence even during treatment with M, B, or O. The consistency of these fragments across treatments also suggests that M does not inhibit archaeal methanogens, which supports earlier theories (Chen and Wolin, 1979). There are, however, several fragments only detected in the B and O treatments. This could be due to inhibition by M or selection by B and O. The fragment size of 203 bp was present after *Rsa*I digestion in C and B, and O had a fragment size of 204 bp, but there was not one of similar size in M. Therefore, M may have an inhibitory affect on the methanogen with this terminal restriction fragment generated after *Rsa*I digestion. Although methane production was lowered upon treatment with all additives (M, B, and O), there were several archaeal fragment patterns across treatments, suggesting that many methanogens remained active.

Sequence Effects

The sequence in which the additives were added did not alter fermentation or microbial cell numbers. However, there appear to be fluctuations in the microbial population due to sequence. The addition of O to M treated fermentors (MO) resulted in lower diversity than the addition of M to the O treatment (OM), but, the MO treatment also had lower fragment utilization. Therefore, MO could result in lower diversity or could increase the number of unique species that have not been studied and sequenced.

The absence of *Gammaproteobacteria* in MO may be explained by the absence of *Pseudomonas* or *Vibrio* fragment patterns in the MO treatment. Searching the Multiple category did not reveal any possible matches in either genera, while OM had both confirmed and possible matches. The difference in *Gammaproteobacteria* in MO and OM could indicate that adding O to M (MO) treatment inhibits some Gram (-) organisms.

The Gram (+) genus *Bacillus* made up more of the total unique species in MO than in OM, which could help explain why MO resulted in more *Bacilli* than OM. *Streptococcus*, another member of the Class, does not provide information that would help explain changes in *Bacilli*.

The unique species in the genera *Actinomyces and Streptomyces* cannot be used to explain the increased number of unique species in *Actinobacteria* in BO compared to OB. On the contrary, no unique species were revealed in BO in *Actinomyces* or *Streptomyces* while they were both present in OB. Therefore, other genera must have experienced greater changes that caused a fluctuation in *Actinobacteria.*

Differences in the *Bacteroides* genus however, could possibly explain the difference between treatments of the *Bacteroidetes* Class, as the number of unique species in both the genus and the Class and seemed to be higher in BO than in OB.

There were no identified species in either *Selenomonas* of *Eubacterium* of BO, but they were present in each genus in OB. When searched within the Multiple classification, the proportion of unique species in OB remains greater than BO. This could partly explain the apparently higher proportion of *Clostridia* after OB treatment than after BO treatment

The greater number of *Bacilli* in OB than BO may also be partially explained by fluctuations in one of its genera. There were no species identified in Bacillus in BO, but unique species in Bacillus accounted for 2% of the species in BO and searching the Multiple classification reveals the possibility that an additional 2% of the species in OB belong to Bacillus. There were no species identified in *Streptococcus*.

The genera *Pseudomonas* had unique species in the OB treatment and *Vibrio* had them in the BO treatment, so changes in these genera cannot be used to explain the higher proportion of *Gammaproteobacteria* in OB than in BO. Searching the Multiple classification for possible species members of the two genera did not change the proportions enough to explain the differences between treatments of *Gammaproteobacteria*. Changes in other Gram (-) organisms in this Class, such as *Ruminobacter*, are likely the cause for the changes in *Gammaproteobacteria* due to sequence.

Concluding Remarks

There were no significant effects of time on fermentation of the C fermentors. The first hypothesis holds, as the effects of M and O on rumen fermentation were similar. Based on information obtained at the Class level, the additives M, B, and O caused population shifts that were different from one another. This is likely due to the different mode of actions of the three additives. It is known that although M and B have similar effects on fermentation, they have different modes of action. Therefore, even though M and O also have similar effects on fermentation, they likely have different modes of action. When studying the mode of action of a supplement in the rumen, it seems to be necessary to look at more than just fermentation parameters.

The second part of the hypothesis holds, as the sequence of adding O and an antibiotic did not affect fermentation, but the sequence did appear to change the microbial population. M dropped fragment utilization, but not if O had already been added. O did not have this affect alone, but it seems to accentuate the affect of M on fragment utilization. The differences in the sequence of M and O addition are likely because of different modes of action of the two additives. When O was added first (OM and OB), there was greater diversity than when the antibiotics were added first (MO and BO).

Roughly half of the unique species in the samples across all treatments are from Unclassified microorganisms and one quarter may belong to closely related species. This shows, along with previous clone library studies, that many of the organisms in the rumen are uncultured (Edwards, et al., 2004, Tajima, et al., 1999, Tajima, et al., 2000, Whitford, et al., 1998).

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Table 1. Trial schedule.

Fermentor	Days 1-4	Days 5-10	Days 11-16
1	C	C	C
2	C	M	MO
3	C	O	OM
4	C	B	BO
5	C	O	OB

Table 2. Usage by InSilico of fragments generated by T-RFLP.

	Utilization of Fragments (%)		
Treatment	*Rsa*I	*Hha*I	*Msp*I
C d4	84	90	98
C d10	78	96	89
C d16	87	93	89
M	79	79	90
B	87	92	98
O	95	95	87
MO	46	85	69
OM	90	95	84
BO	75	100	86
OB	92	87	93

Table 3. The effect of the additives monensin (M), bacitracin (B), and oil (O) and the sequence in which they are added (MO vs. OM) and (BO vs. OB) on VFA production.

		Additive			Sequence				
	Control				M and O		B and O		
	C	M	B	O	MO	OM	BO	OB	SE
Total VFA, mM[1]	49.8	49.2	52.8	48.6	59.8	60.3	67.9	63.9	7.17
Individual VFA, mM									
Acetate (A)	34.1	30.2	34.7	30.1	35.1	35.6	41.7	39.0	5.86
Propionate (P)	9.4	11.0	9.8	10.3	14.5	15.1	15.4	14.9	1.98
Butyrate	4.1^{a}	5.2^{ab}	5.6^{b}	5.2^{b}	6.6	6.0	7.1	6.7	0.60
Isobutyrate	0.4	0.3	0.4	0.4	0.4	0.4	0.5	0.5	0.04
Valerate	0.8^{a}	1.0^{ab}	1.1^{ab}	1.1^{b}	1.4	1.6	1.6	1.4	0.20
Isovalerate	0.9^{a}	1.4^{ab}	1.3^{ab}	1.5^{b}	1.7	1.6	1.7	1.5	0.26
Individual VFA, mol/100 mol									
Acetate (A)	68.1^{e}	60.5^{f}	65.3^{ef}	61.4^{f}	58.4	58.3	61.3	60.7	4.11
Propionate (P)	19.2^{a}	22.8^{b}	18.6^{ac}	21.4^{bc}	24.4	25.4	22.6	23.5	2.67
Butyrate	8.3^{e}	10.8^{f}	10.8^{f}	11.0^{f}	11.2	10.2	10.5	10.5	0.96
Isobutyrate	0.8	0.8	0.8	0.9	0.8	0.7	0.7	0.7	0.11
Valerate	1.7^{e}	2.1^{f}	2.1^{f}	2.3^{f}	2.3	2.7	2.4	2.2	0.31
Isovalerate	1.9^{a}	3.1^{b}	2.6^{ab}	3.1^{b}	2.9	2.7	2.5	2.4	0.53
A:P	3.6^{a}	2.7^{b}	3.6^{ac}	3.0^{bc}	2.4	2.5	3.1	2.7	0.60

^{a,b,c,d} Means with superscript differ $p<.05$
^{e,f,g,h} Means with superscript differ $p<.01$
[1] Means with superscript differ $p<.01$

Table 4. The effect of the additives monensin (M), bacitracin (B), and oil (O) and the sequence in which they are added (MO vs. OM) and (BO vs. OB) on total cell numbers, methane production, culture pH, and ammonia concentration.

		Additive			Sequence				
	Control				M and O		B and O		
	C	M	B	O	MO	OM	BO	OB	SE
Cells/mL (billions)	1.4	1.3	1.3	1.3	1.0	1.1	1.1	1.5	0.16
Methane, mmol/d	30.1[a]	17.9[b]	24.8[c]	19.4[b]	18.9	19.0	21.9	19.5	2.89
Culture pH[1]	6.0[e]	6.2[f]	6.1[ef]	5.9[g]	6.0*	5.7	5.9	5.8	0.06
NH_3, mg/100ml	23.8	22.0	22.0	22.7	24.6	23.9	23.4	24.8	1.63
Digestibility, %	43.0	38.4	43.3	38.3	45.6	45.5	51.9	48.5	5.20

[a,b,c,d] Means with superscript differ $p<.05$
[e,f,g,h] Means with superscript differ $p<.01$
[1] Means with superscript differ $p<.01$

Table 5. Comparison of T-RFLP from control (A), additive (B), and sequence (C) treatments across three replicates. Samples were amplified with a bacterial universal primer and digested with *Rsa*I, *Hha*I, and *Msp*I. (*indicates 4 PCR reactions performed)

A.

	C d4			C d10			C d16		
Replicate	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I
Rep 1	22	20	24	11	17	26	8	12	21
Rep 2	14	20	19	13	18	21	12	20	19
Rep 3	17	10	20	12	15	20	12	14	15
Fragments in all reps	10	9	14	9	12	15	6	10	13
Fragments in both Rep 1 and Rep 2	12	14	17	9	15	19	8	12	17
Fragments in both Rep 2 and Rep 3	11	10	15	10	13	17	8	12	13
Fragments in both Rep 1 and Rep 3	12	11	16	9	12	16	6	10	13

B.

	C			M			B			O		
Replicate	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I
Rep 1	22	20	24	19	22	16	17	22	28	9	16	26
Rep 2	14	20	19	15	8	19	13	12	12	30*	32*	32*
Rep 3	17	10	20	5	10	6	14	10	15	16	15	20
Fragments in all reps	10	9	14	5	5	6	8	8	9	7	10	11
Fragments in both Rep 1 and Rep 2	12	14	17	12	7	10	10	12	11	9	14	25
Fragments in both Rep 2 and Rep 3	11	10	15	4	6	6	9	8	9	15	13	18
Fragments in both Rep 1 and Rep 3	12	11	16	5	8	6	11	10	14	7	10	11

C.

	MO			OM			BO			OB		
Replicate	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I	*Rsa*I	*Hha*I	*Msp*I
Rep 1	29	12	23	10	14	17	15	10	22	11	21	26
Rep 2	21	19	26	10	17	18	4	10	17	12	13	18
Rep 3	11	13	18	17	19	19	19	14	14	11	14	19
Fragments in all reps	9	7	11	7	9	11	3	5	7	6	9	11
Fragments in both Rep 1 and Rep 2	15	12	18	9	10	14	3	6	11	6	10	13
Fragments in both Rep 2 and Rep 3	10	8	16	8	12	14	4	6	8	6	9	13
Fragments in both Rep 1 and Rep 3	9	7	12	8	9	13	8	8	11	9	13	16

Table 6. Comparison of T-RFLP from control (A), additive (B), and sequence (C) treatments within the first replicate. Samples were amplified with a bacterial universal primer and digested with *Rsa*I, *Hha*I, and *Msp*I.

A.

Fermentor Sample	***Rsa*I**	***Hha*I**	***Msp*I**
C d4	21	24	24
C d10	12	16	28
C d16	8	11	23
Fragments in all samples	6	9	17
Fragments in both C d4 and C d10	10	15	21
Fragments in both C d10 and C d16	8	9	20
Fragments in both C d4 and C d16	8	10	18

B.

Fermentor Sample	***Rsa*I**	***Hha*I**	***Msp*I**
C	21	24	24
M	18	20	16
B	14	23	25
O	9	17	31
Fragments in both C and M	13	15	11
Fragments in both C and B	13	17	19
Fragments in both C and O	9	16	20
Fragments in both M and B	9	16	12
Fragments in both M and O	8	11	15
Fragments in both B and O	9	14	22

C.

Fermentor Sample	***Rsa*I**	***Hha*I**	***Msp*I**
MO	28	12	19
OM	9	13	17
BO	26	11	23
OB	12	22	25
Fragments in both MO and OM	8	7	12
Fragments in both BO and OB	8	9	20

Table 7. Comparison of T-RFLP from control (A), additive (B), and sequence (C) treatments within the first replicate. Samples were amplified with an archaeal universal primer and digested with *Rsa*I, *Hha*I, and *Msp*I.

A.

Fermentor Sample	***Rsa*I**	***Hha*I**	***Msp*I**
C d4*	7	5	11
C d10	9	5	13
C d16	8	4	7
Fragments in all samples	6	0	5
Fragments in both C d4 and C d10	6	4	5
Fragments in both C d10 and C d16	6	0	6
Fragments in both C d4 and C d16	5	0	5

B.

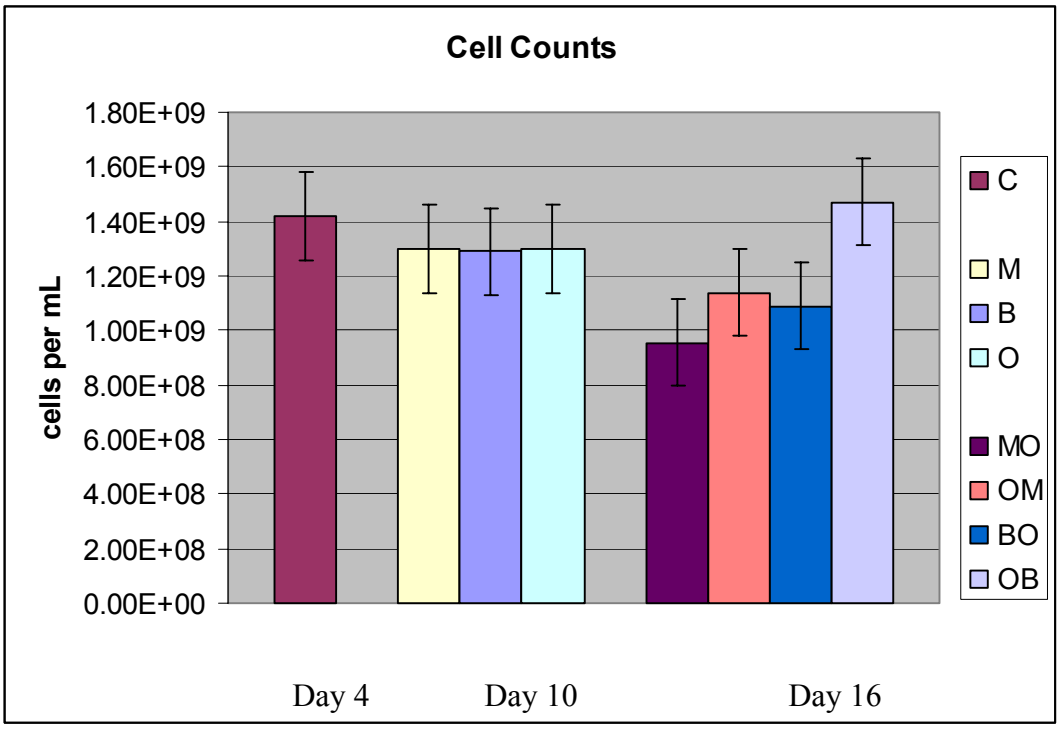
Fermentor Sample	***Rsa*I**	***Hha*I**	***Msp*I**
C*	7	4	7
M	8	9	7
B	13	8	12
O	9	13	11
Fragments in both C and M	6	4	5
Fragments in both C and B	7	4	6
Fragments in both C and O	5	4	5
Fragments in both M and B	7	7	6
Fragments in both M and O	6	7	6
Fragments in both B and O	5	7	7

C.

Fermentor Sample	***Rsa*I**	***Hha*I**	***Msp*I**
MO	7	6	7
OM	7	8	5
BO	5	5	7
OB	9	7	9
Fragments in both MO and OM	6	4	5
Fragments in both BO and OB	4	4	6

*indicates additional PCR reactions performed

A.



B.

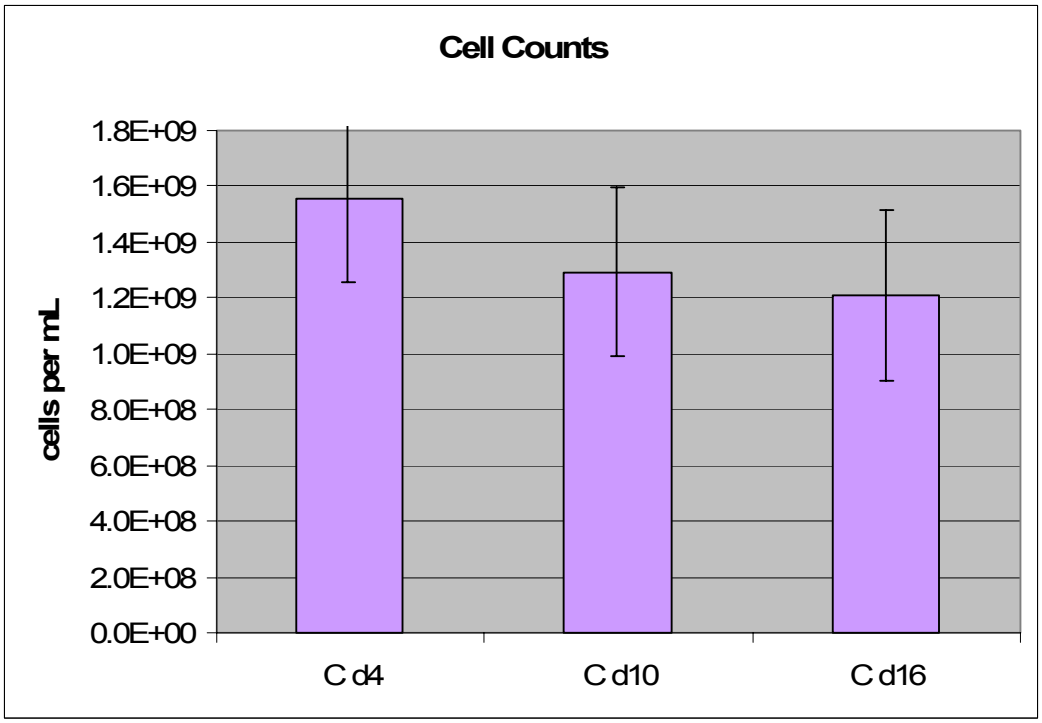
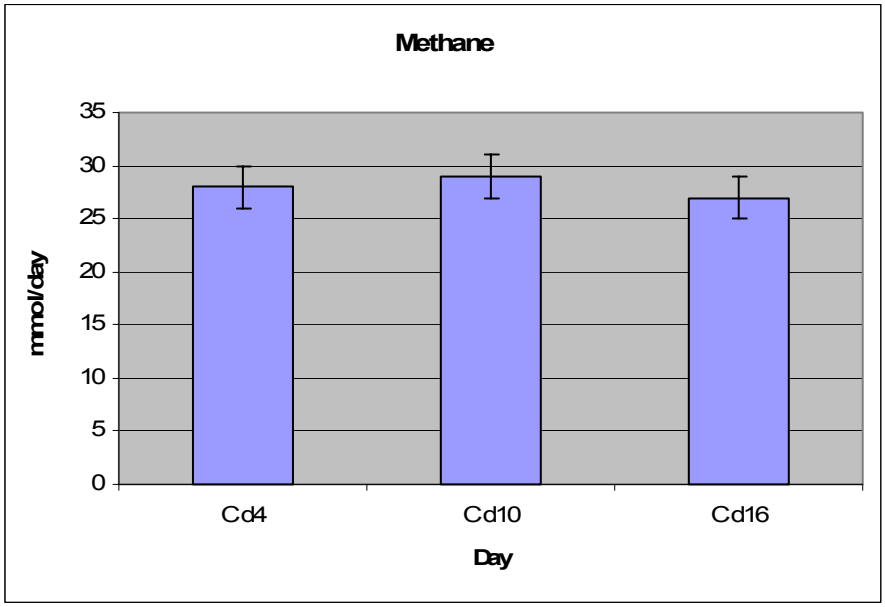


Figure 1. Cell counts of treatment (A) and control (B) fermentors.

A.



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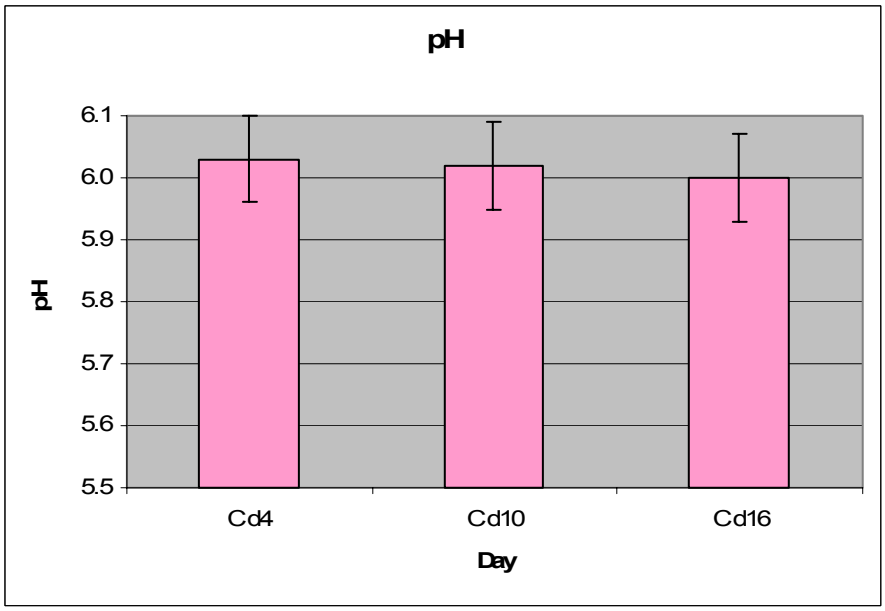
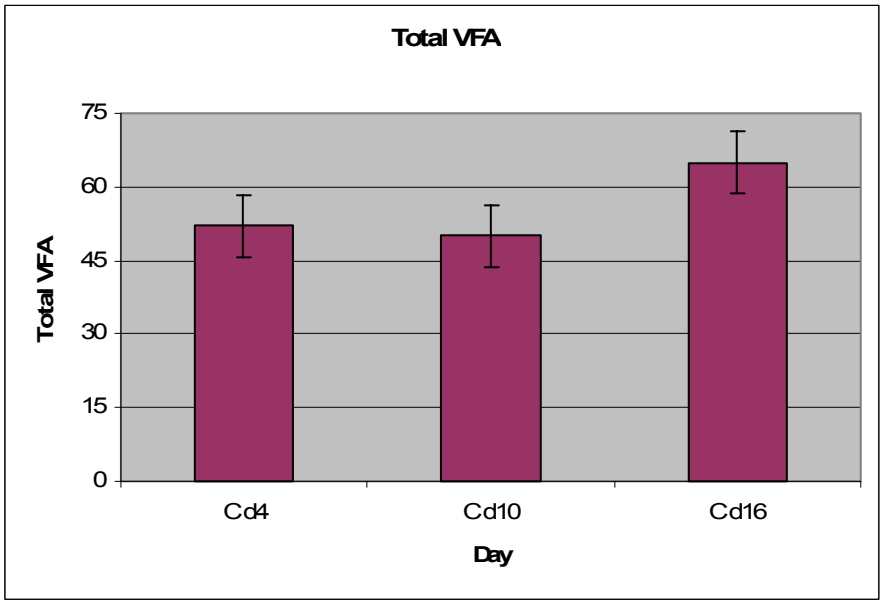


Figure 2. Effect of time on the methane production (A) and pH (B) of the control fermentors.

A.



B.

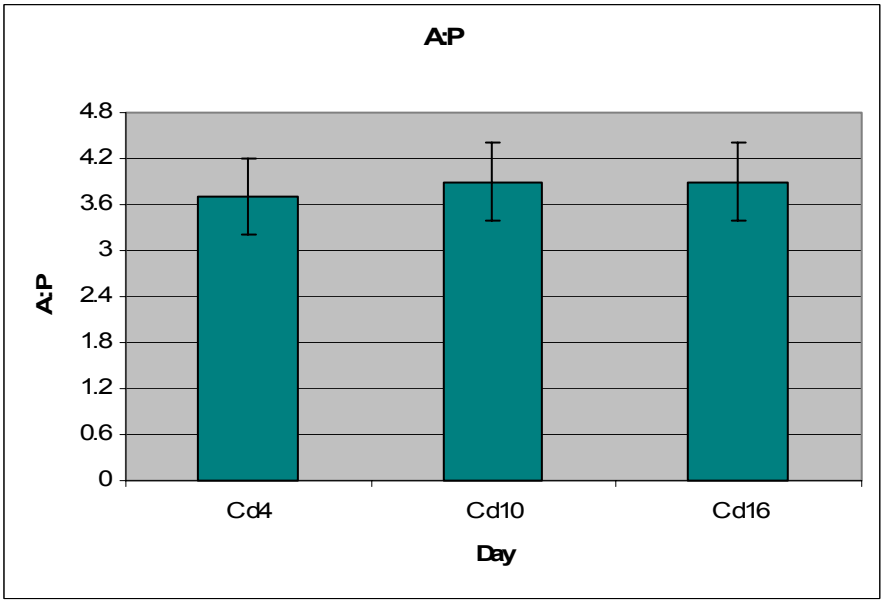


Figure 3. Effect of time on total VFA production (A) and A:P (B) of the control fermentors.

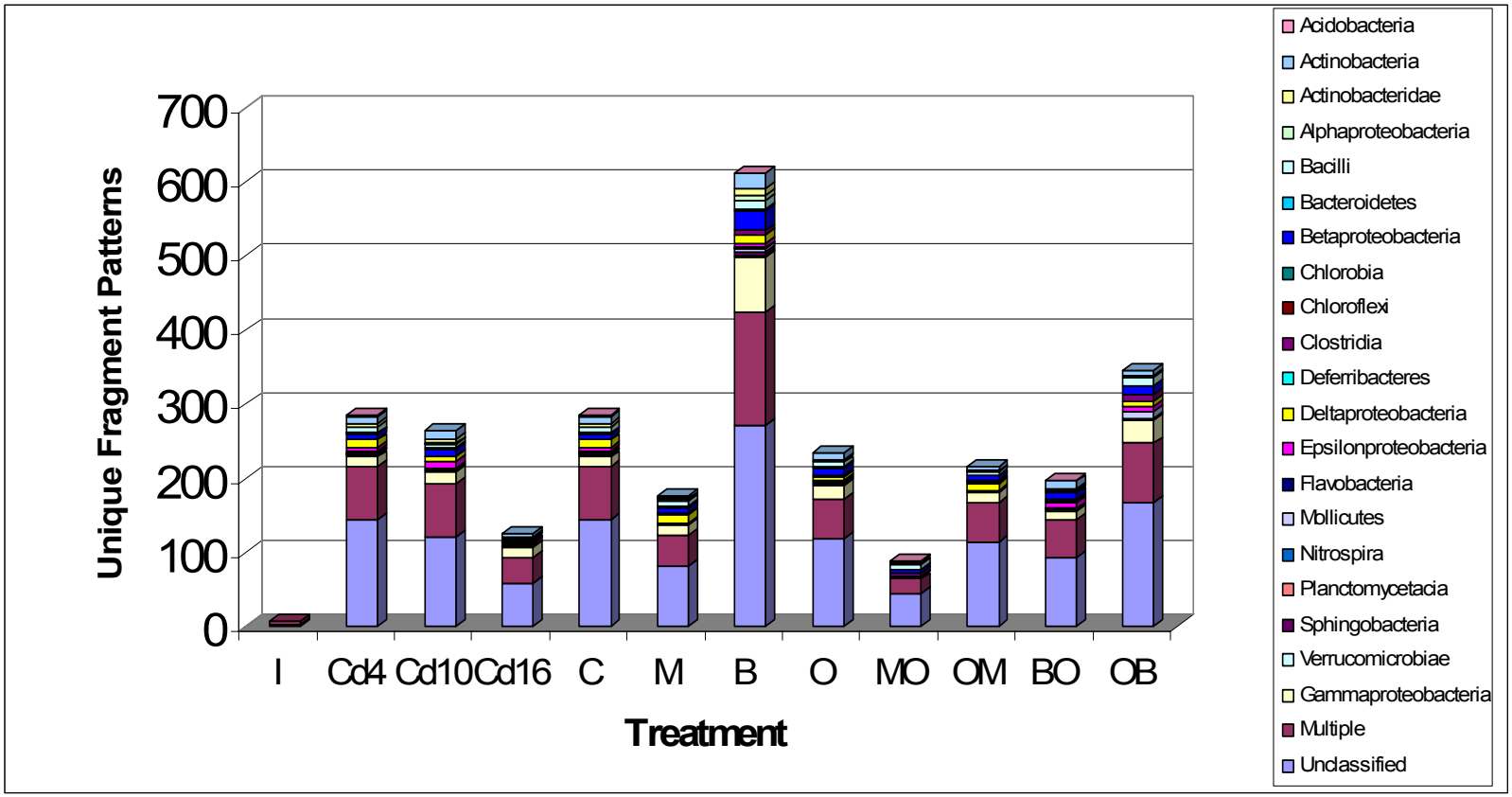


Figure 4. Graphical representation of the number of unique species identified per class of control and treatments.

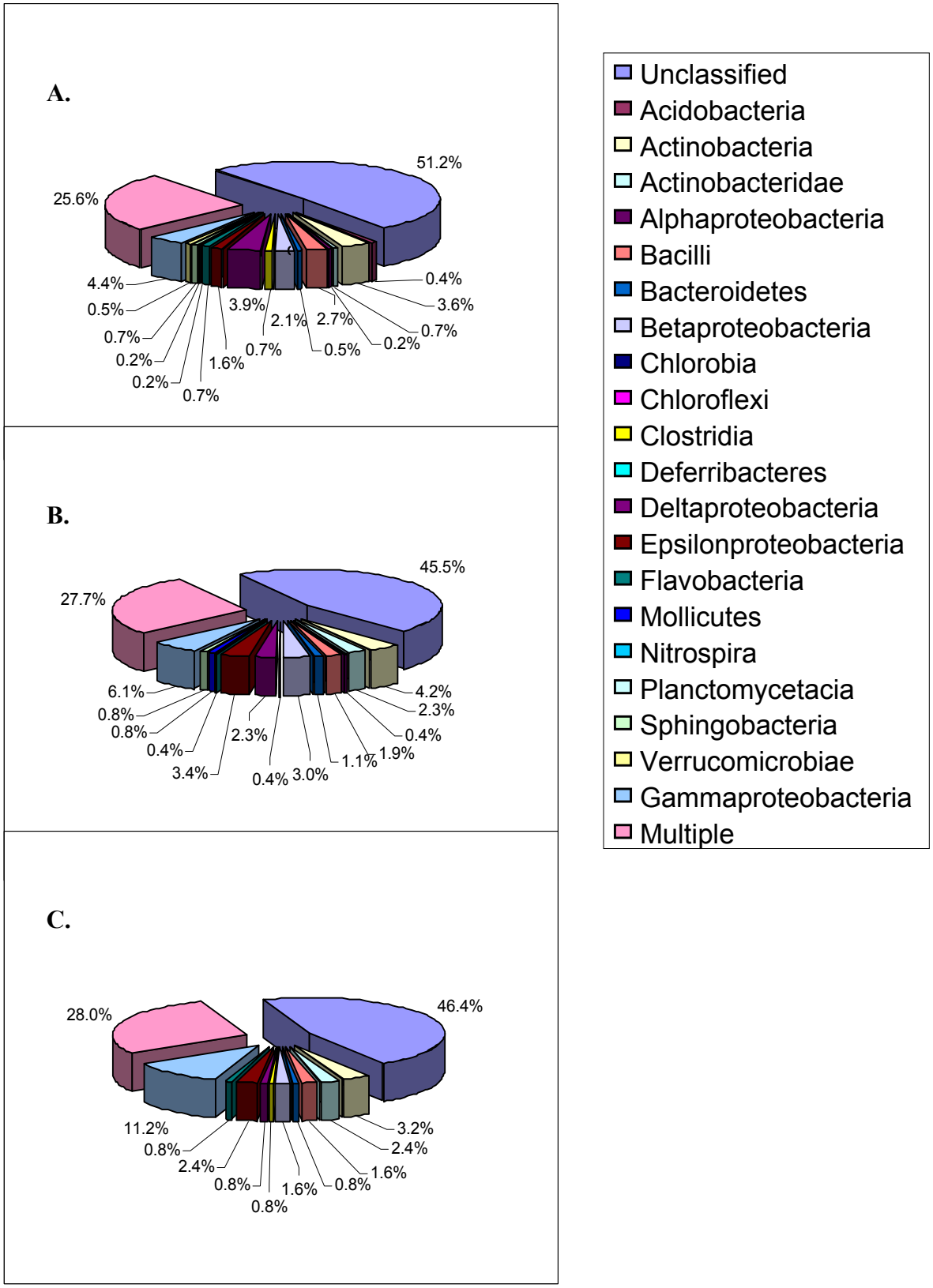


Figure 5. Graphical representation of the percentage of unique species identified per class of control on day 4 (A), 10 (B), and 16 (C).

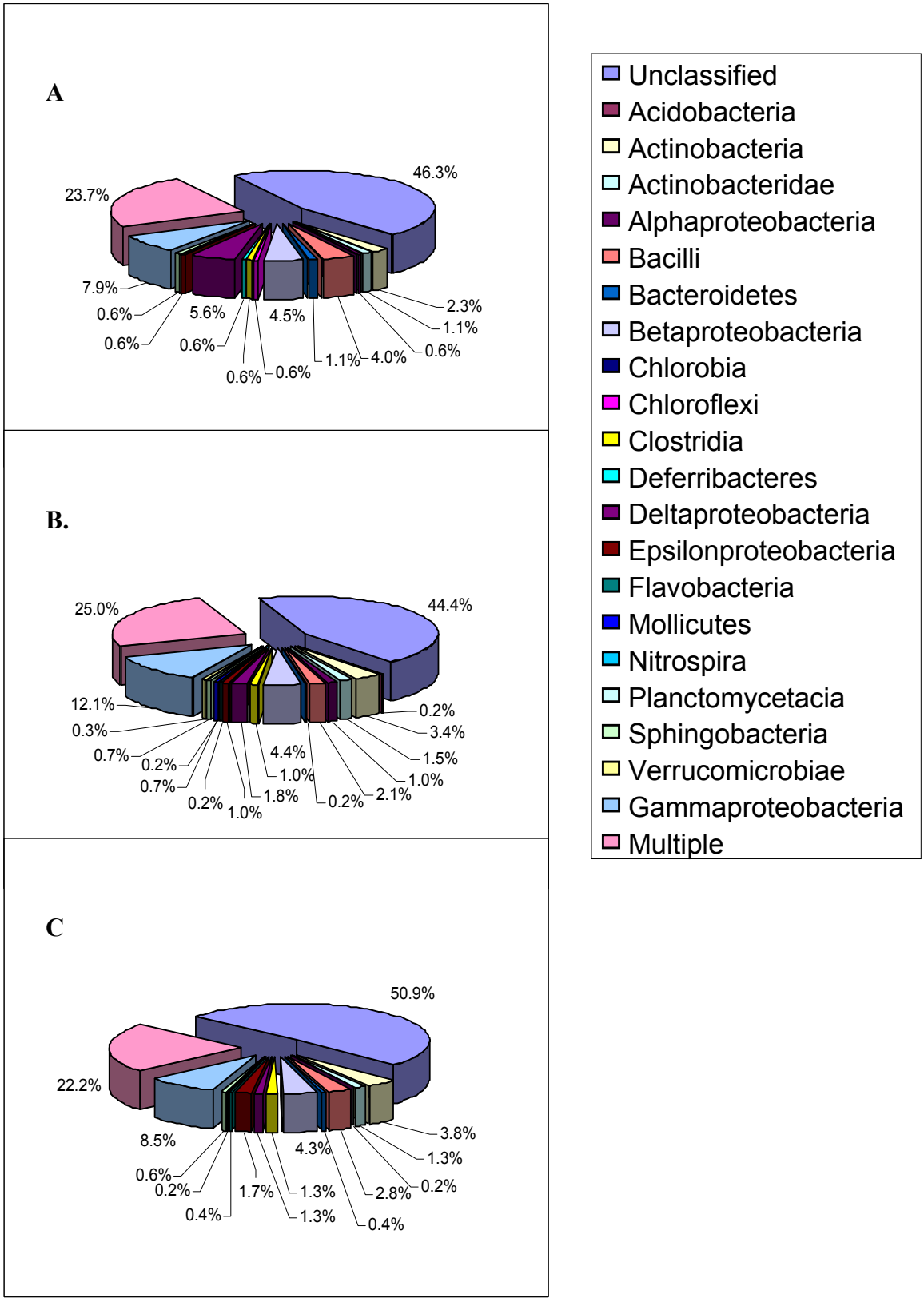


Figure 6. Graphical representation of the percentage of unique species identified per class of additive effects monensin (A), bacitracin (B), and oil (C).

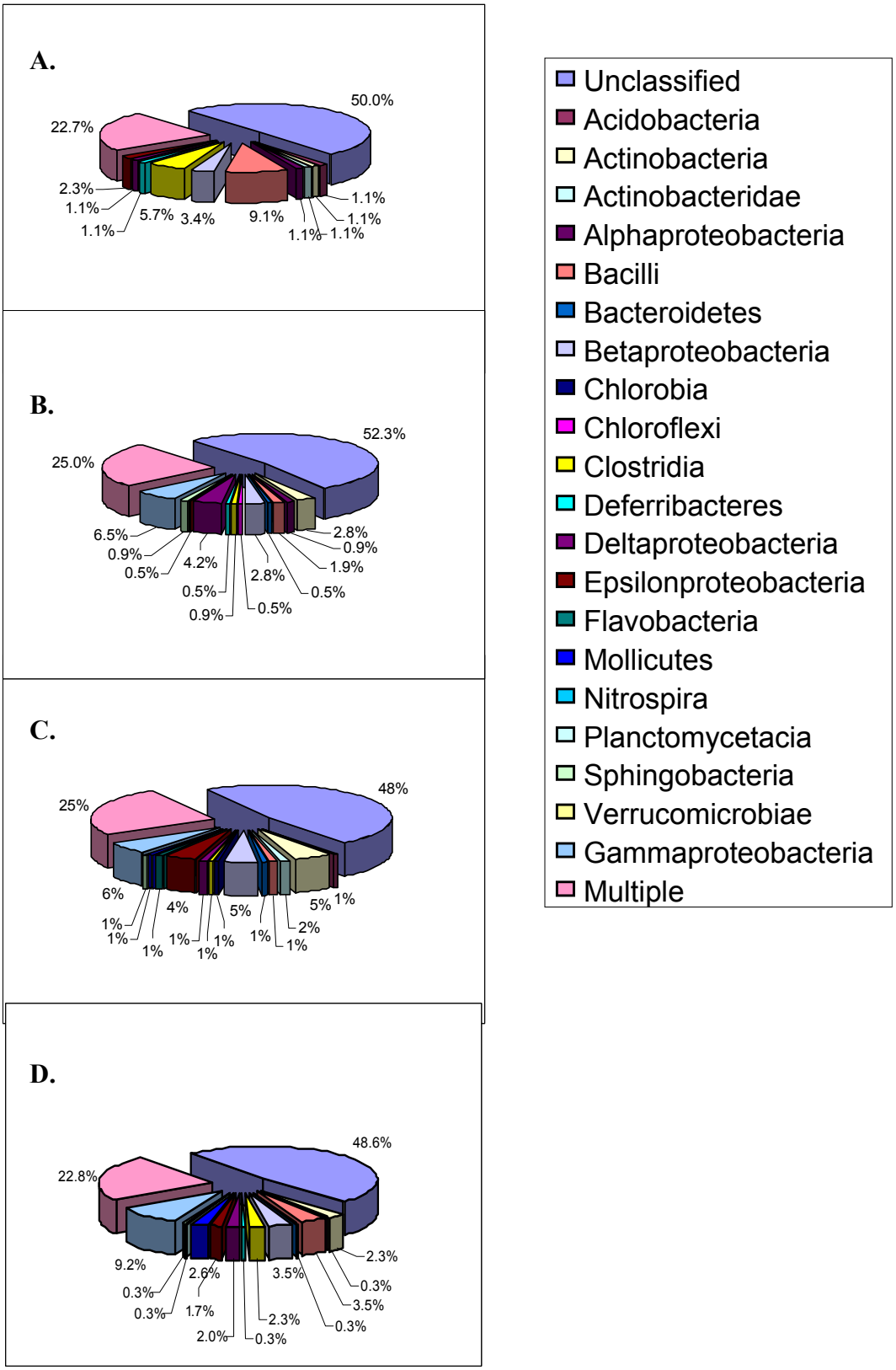
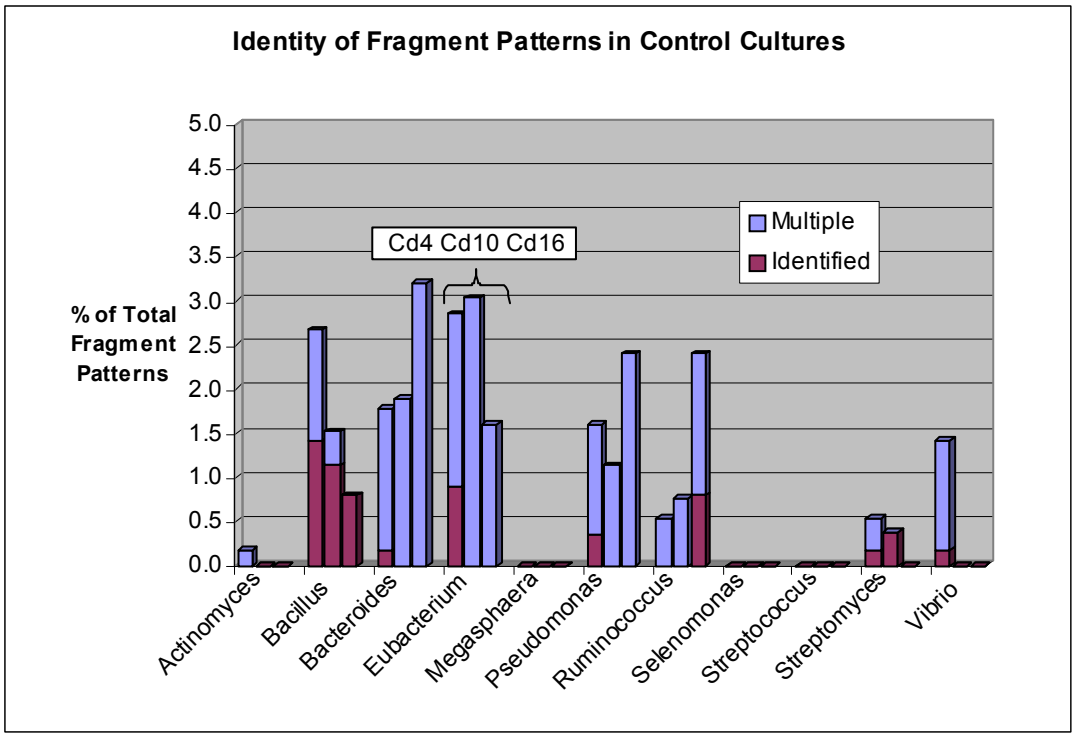


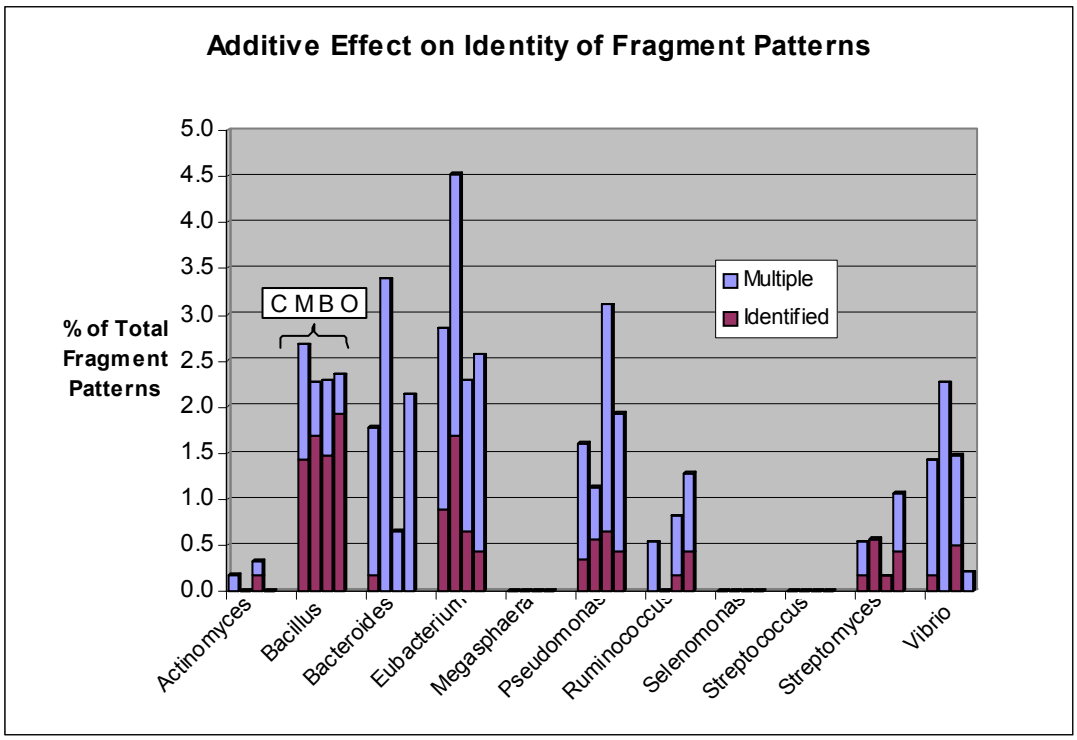
Figure 7. Graphical representation of the percentage of unique species identified per class of sequence effects MO (A), OM (B), BO (C), and OB (D).

Figure 8. The unique fragment patterns of eleven important rumen genera found in the control (A), additive (B), and sequence (C) samples. The identified species are unique fragments assigned to only one genus, and the Multiple category includes fragment patterns that may belong to more than one genus.

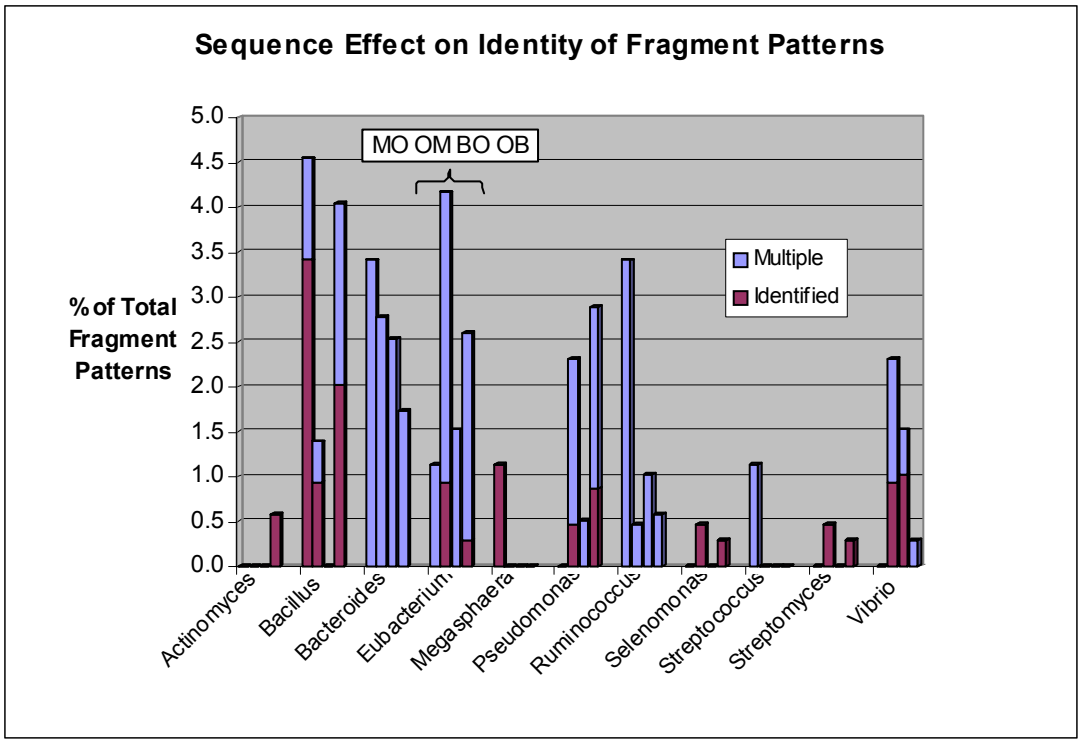
A.


Identity of Fragment Patterns in Control Cultures
% of Total Fragment Patterns
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cd4 Cd10 Cd16
Multiple
Identified
Actinomyces
Bacillus
Bacteroides
Eubacterium
Megasphaera
Pseudomonas
Ruminococcus
Selenomonas
Streptococcus
Streptomyces
Vibrio

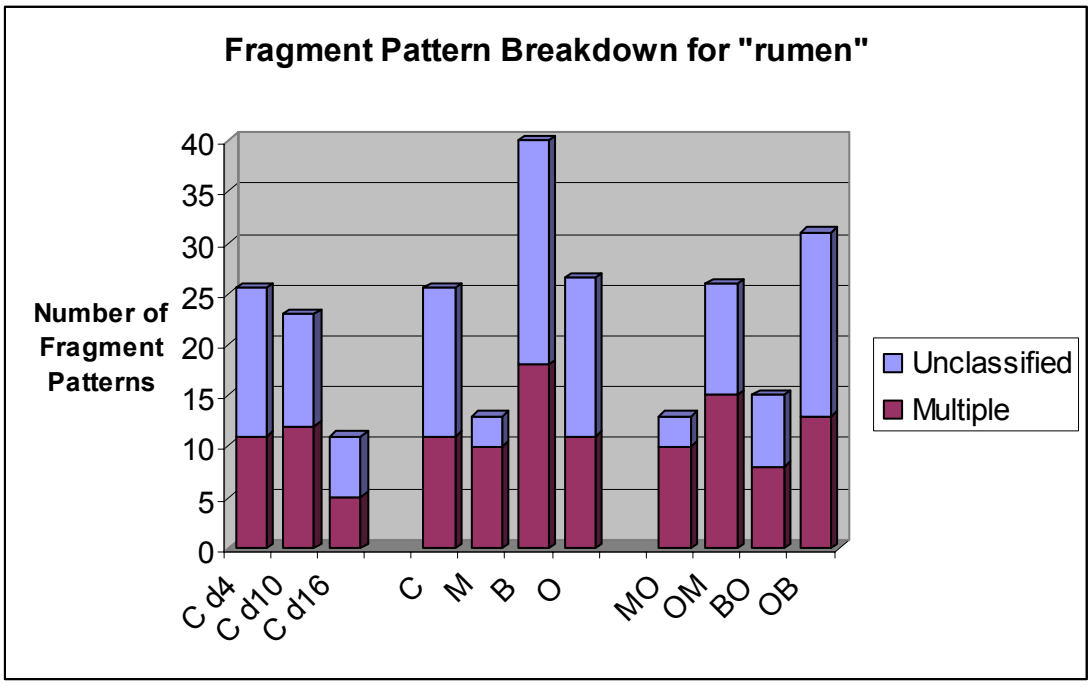
B.


Additive Effect on Identity of Fragment Patterns
% of Total Fragment Patterns
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C M B O
Multiple
Identified
Actinomyces
Bacillus
Bacteroides
Eubacterium
Megasphaera
Pseudomonas
Ruminococcus
Selenomonas
Streptococcus
Streptomyces
Vibrio

C.


Sequence Effect on Identity of Fragment Patterns
% of Total Fragment Patterns
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MO OM BO OB
Multiple
Identified
Actinomyces
Bacillus
Bacteroides
Eubacterium
Megasphaera
Pseudomonas
Ruminococcus
Selenomonas
Streptococcus
Streptomyces
Vibrio

A.



B.

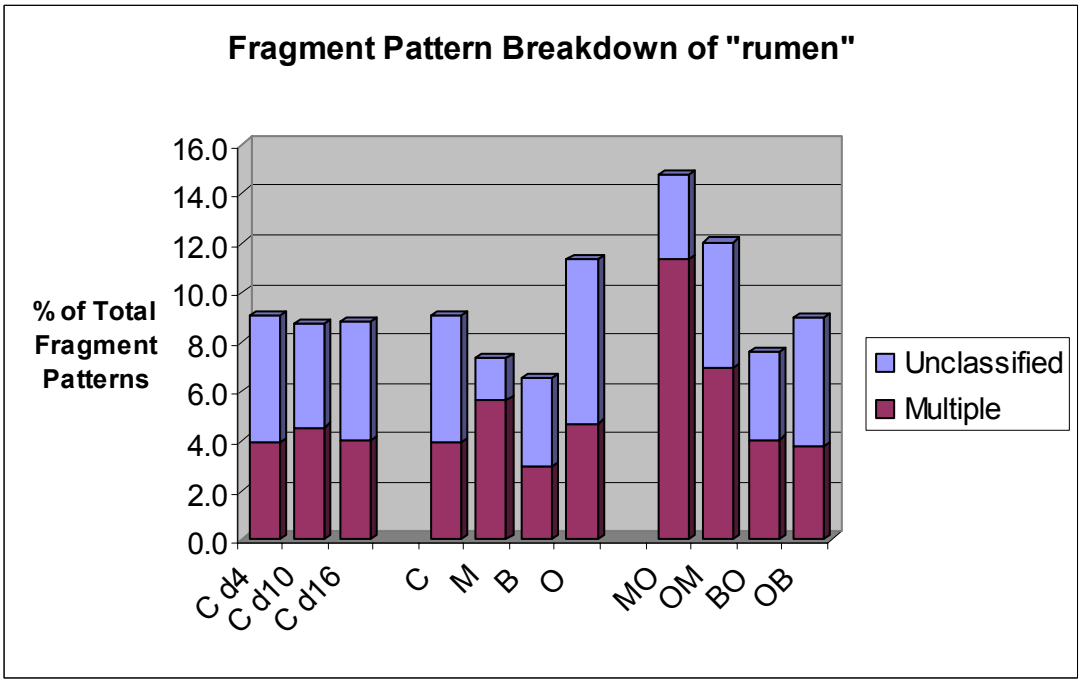
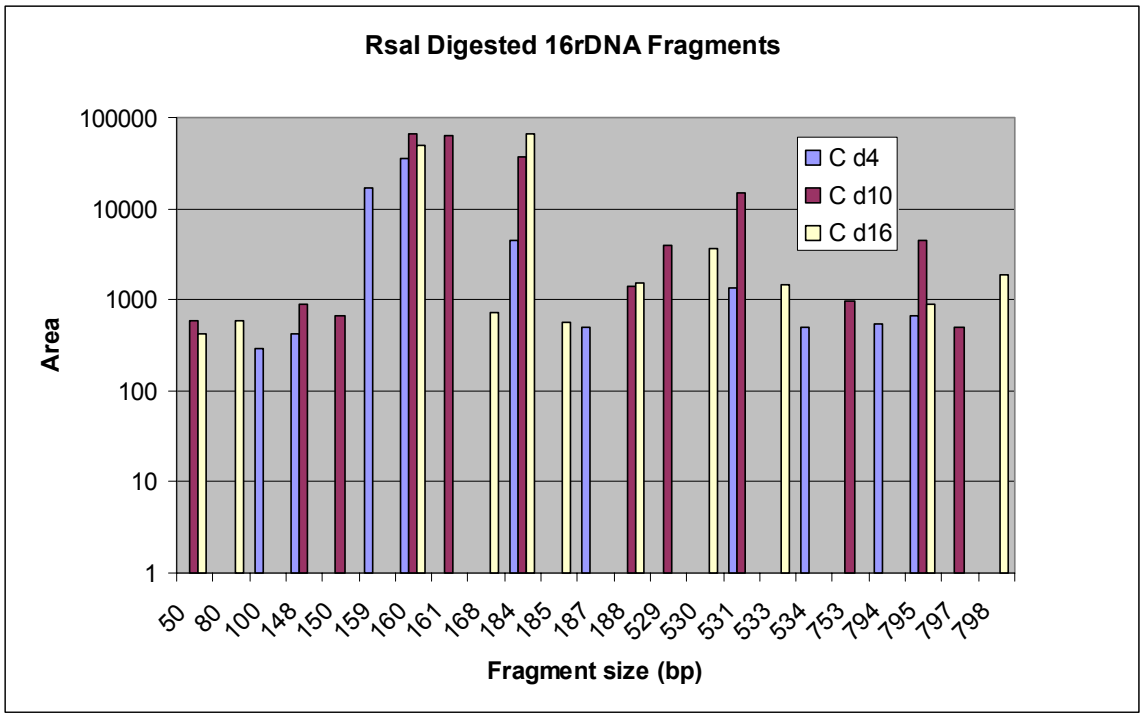


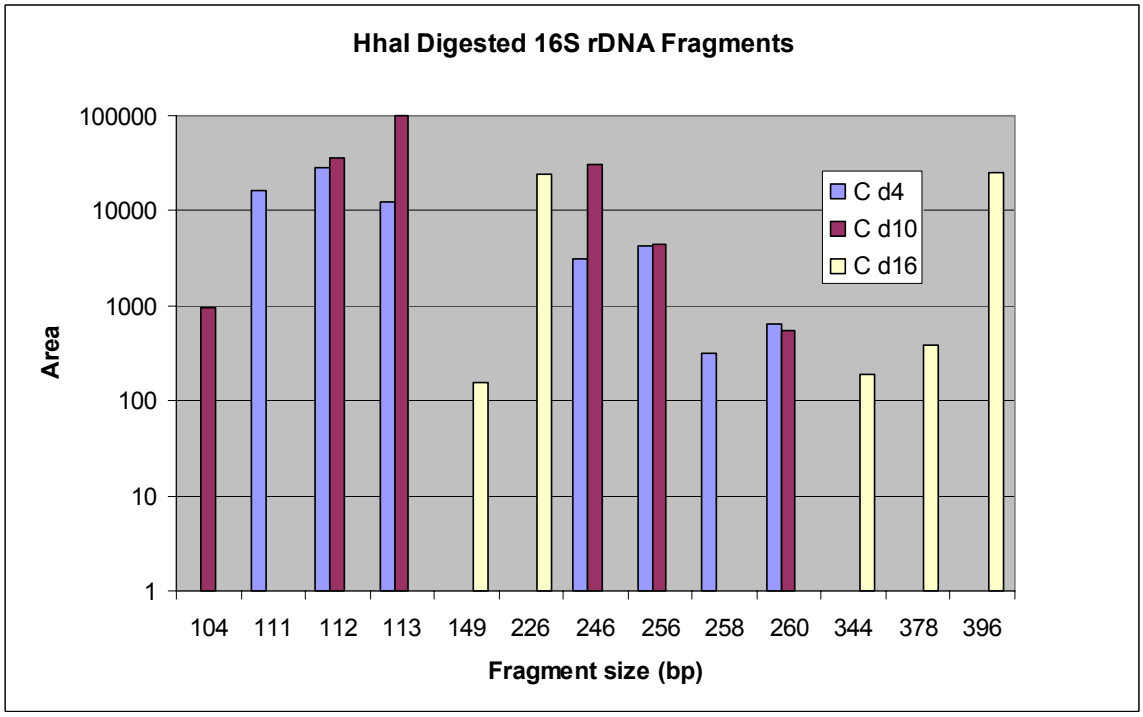
Figure 9. The unique fragment patterns of all treatments associated with "rumen" by number (A) and percentage (B) of total unique fragment patterns. The Unclassified species are unique fragments assigned only to Uncultured and Unidentified Rumen Bacterium. The Multiple category includes fragment patterns that may belong to either an unclassified rumen bacterium, a sequenced bacterium, or both.

Figure 10. Distribution of the T-RFLP of the control samples. 16S rDNA was amplified using an archaeal primer and subsequently digested by RsaI (A), HhaI, (B), and MspI (C).

A.


RsaI Digested 16rDNA Fragments
100000
10000
1000
100
10
1
Area
C d4
C d10
C d16
50 80 100 148 150 159 160 161 168 184 185 187 188 529 530 531 533 534 753 794 795 797 798
Fragment size (bp)

B.


HhaI Digested 16S rDNA Fragments
100000
10000
1000
100
10
1
Area
C d4
C d10
C d16
104 111 112 113 149 226 246 256 258 260 344 378 396
Fragment size (bp)

C.

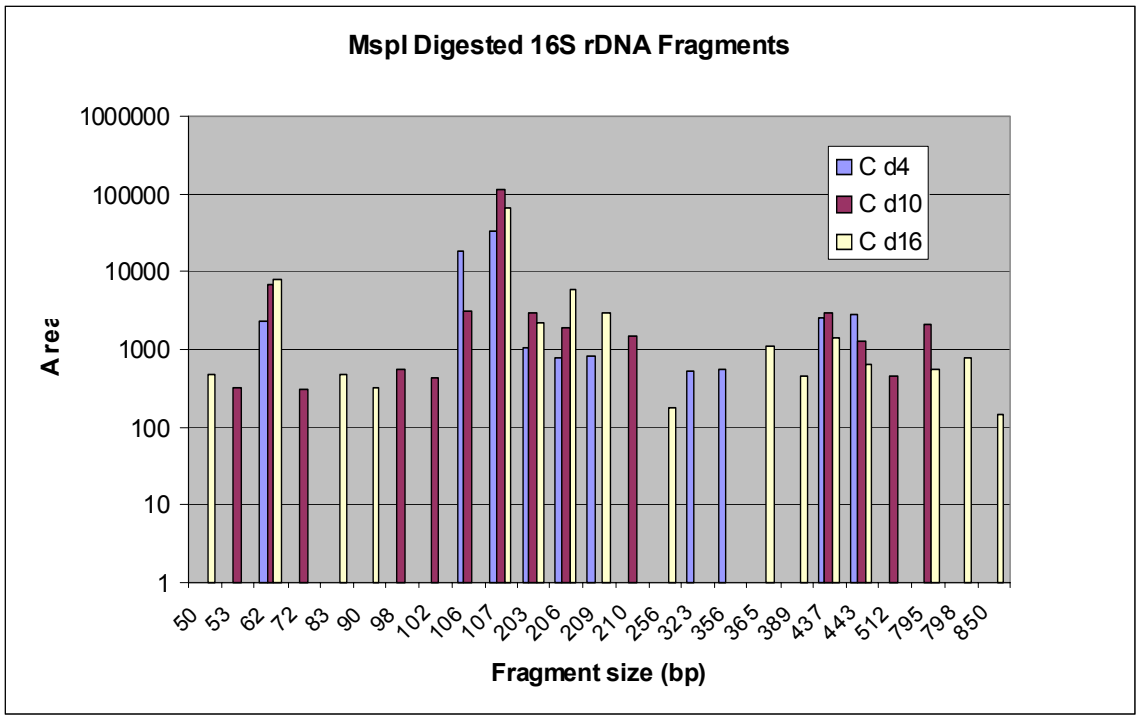
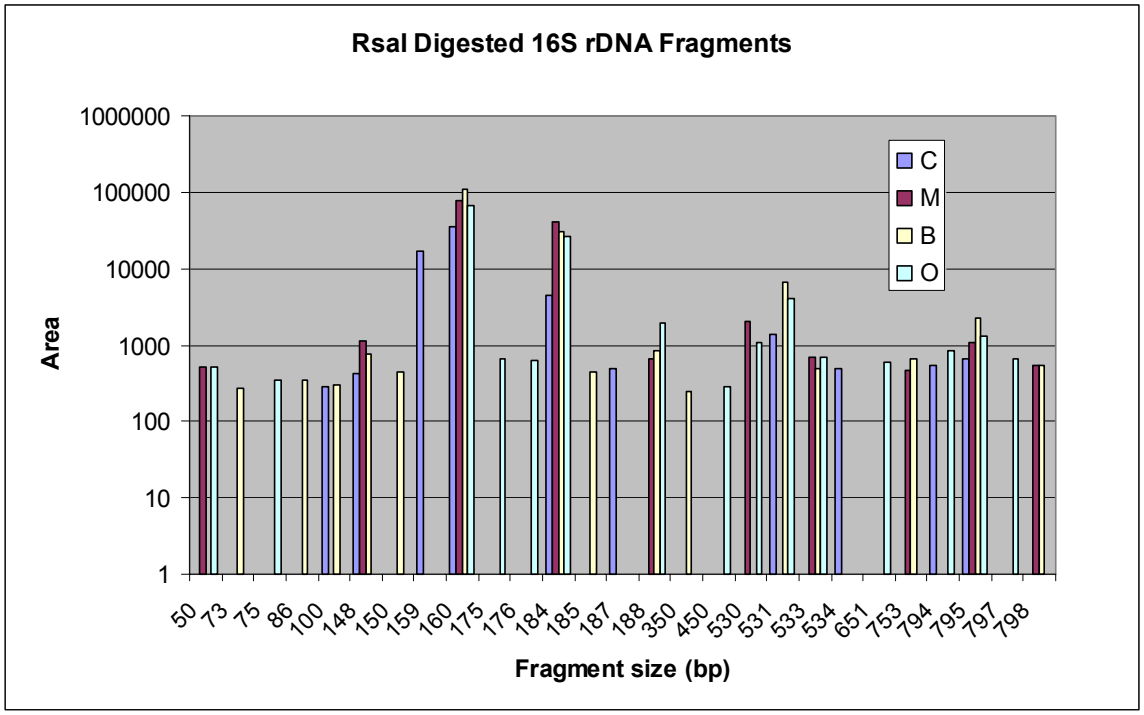
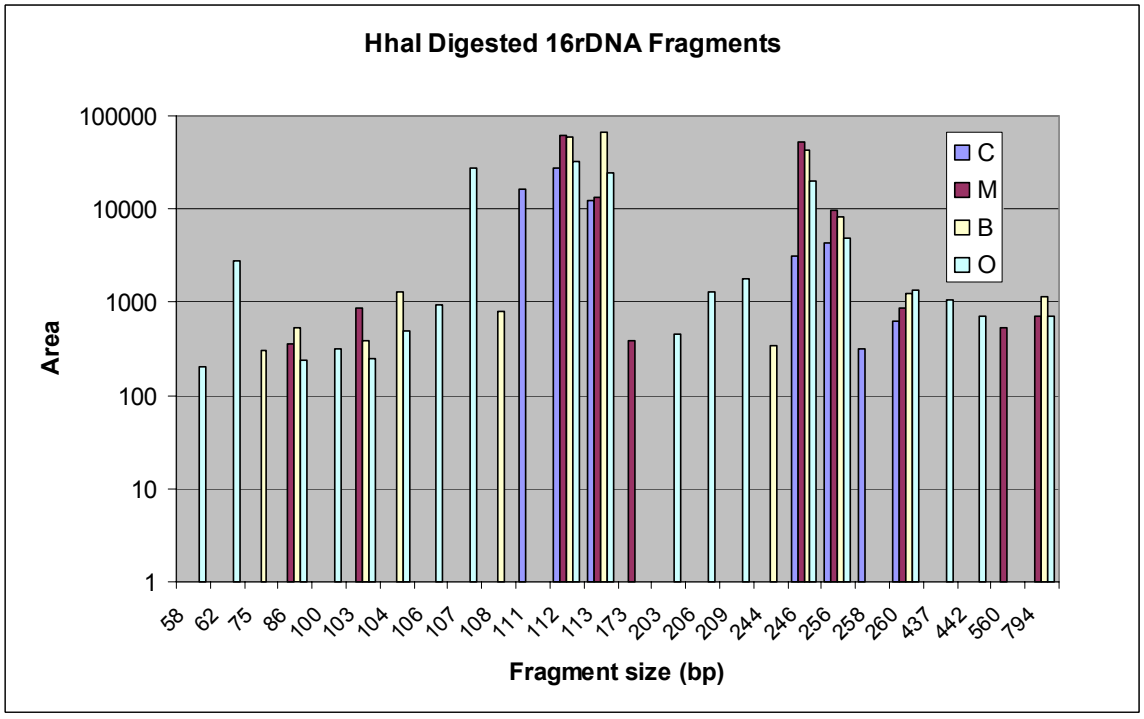

MspI Digested 16S rDNA Fragments
1000000
100000
10000
1000
100
10
1
Area
C d4
C d10
C d16
50
53
62
72
83
90
98
102
106
107
203
206
209
210
256
323
356
365
389
437
443
512
795
798
850
Fragment size (bp)

Figure 11. Distribution of the T-RFLP of the additive samples. 16S rDNA was amplified using an archaeal primer and subsequently digested by RsaI (A), HhaI, (B), and MspI (C).

A.


RsaI Digested 16S rDNA Fragments
1000000
100000
10000
1000
100
10
1
Area
C
M
B
O
50 73 75 86 100 148 150 159 160 175 176 184 185 187 188 350 450 530 531 533 534 651 753 794 795 797 798
Fragment size (bp)

B.


HhaI Digested 16rDNA Fragments
100000
10000
1000
100
10
1
Area
C
M
B
O
58 62 75 86 100 103 104 106 107 108 111 112 113 173 203 206 209 244 246 256 258 260 437 442 560 794
Fragment size (bp)

C.

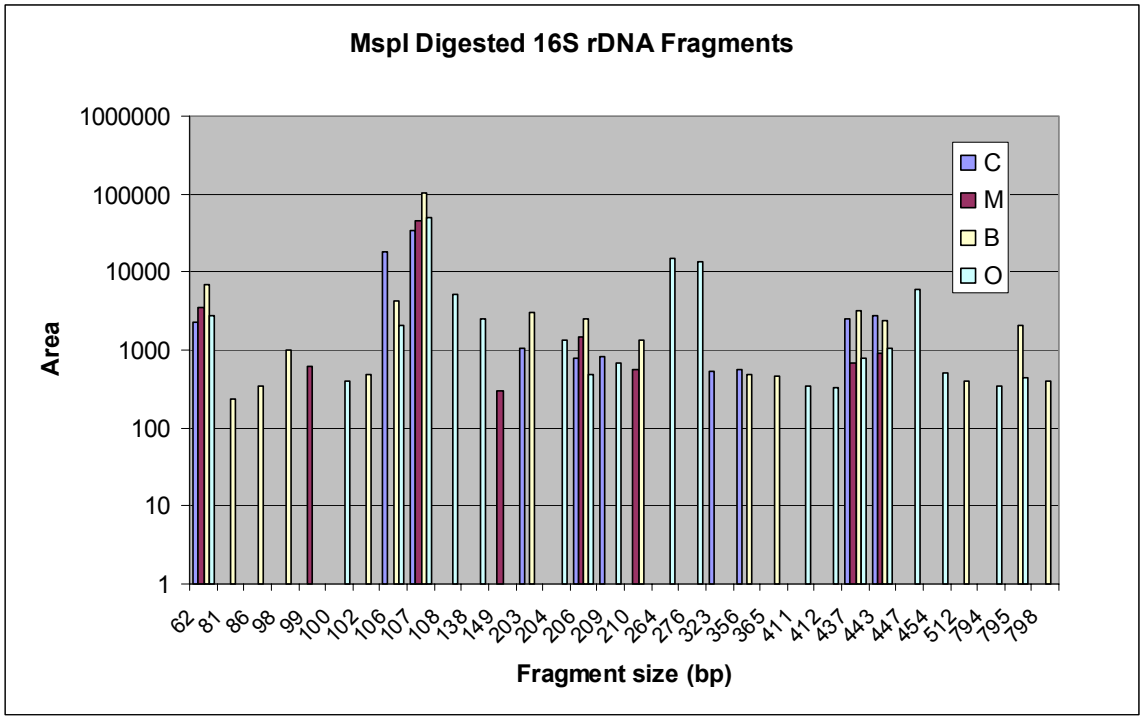
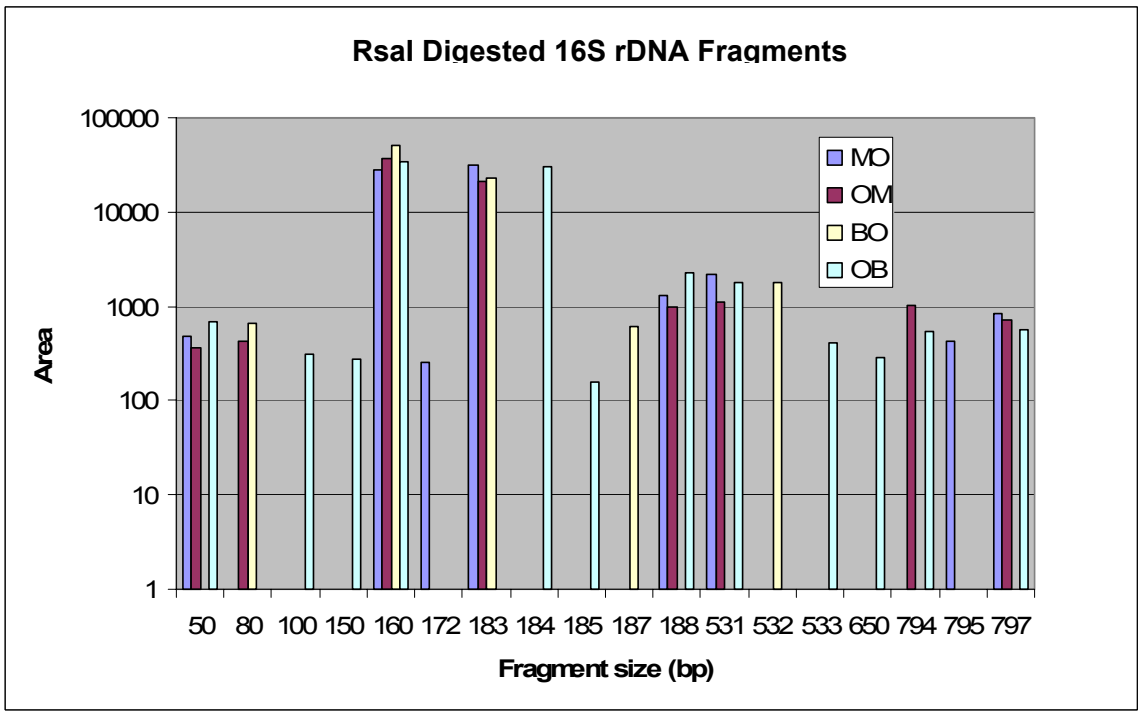
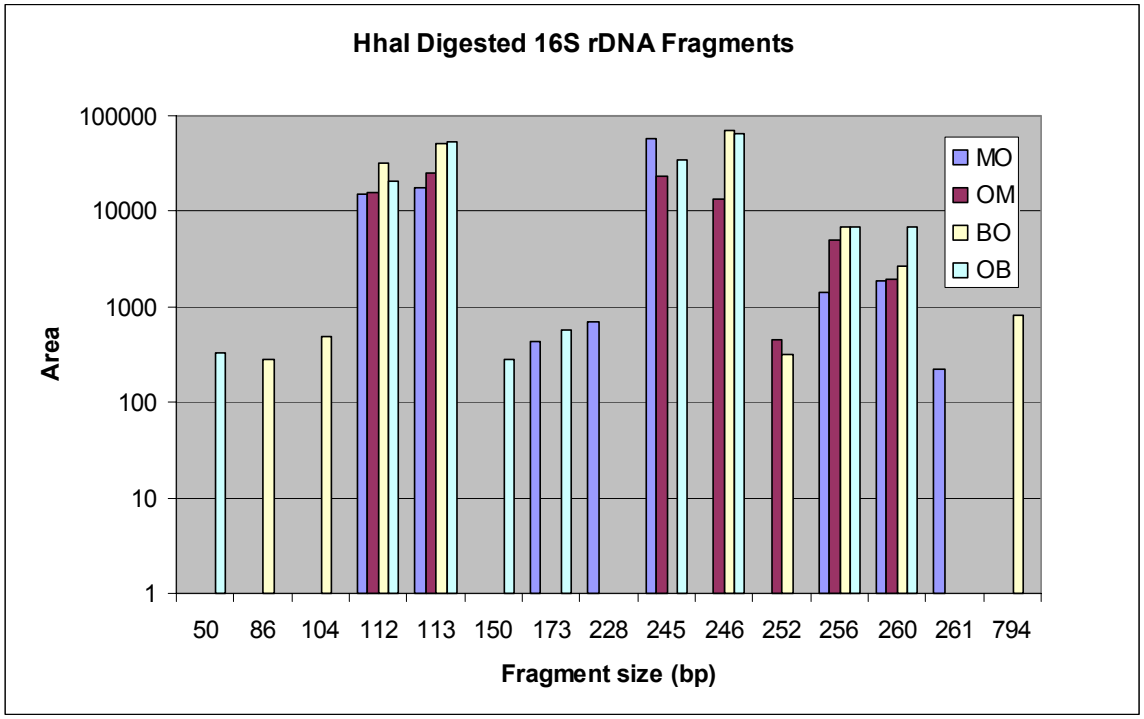

MspI Digested 16S rDNA Fragments
1000000
100000
10000
1000
100
10
1
Area
C
M
B
O
62
81
86
98
99
100
102
106
107
108
138
149
203
204
206
209
210
264
276
323
356
365
411
412
437
443
447
454
512
794
795
798
Fragment size (bp)

Figure 12. Distribution of the T-RFLP of the sequence samples. 16S rDNA was amplified using an archaeal primer and subsequently digested by RsaI (A), HhaI, (B), and MspI (C).

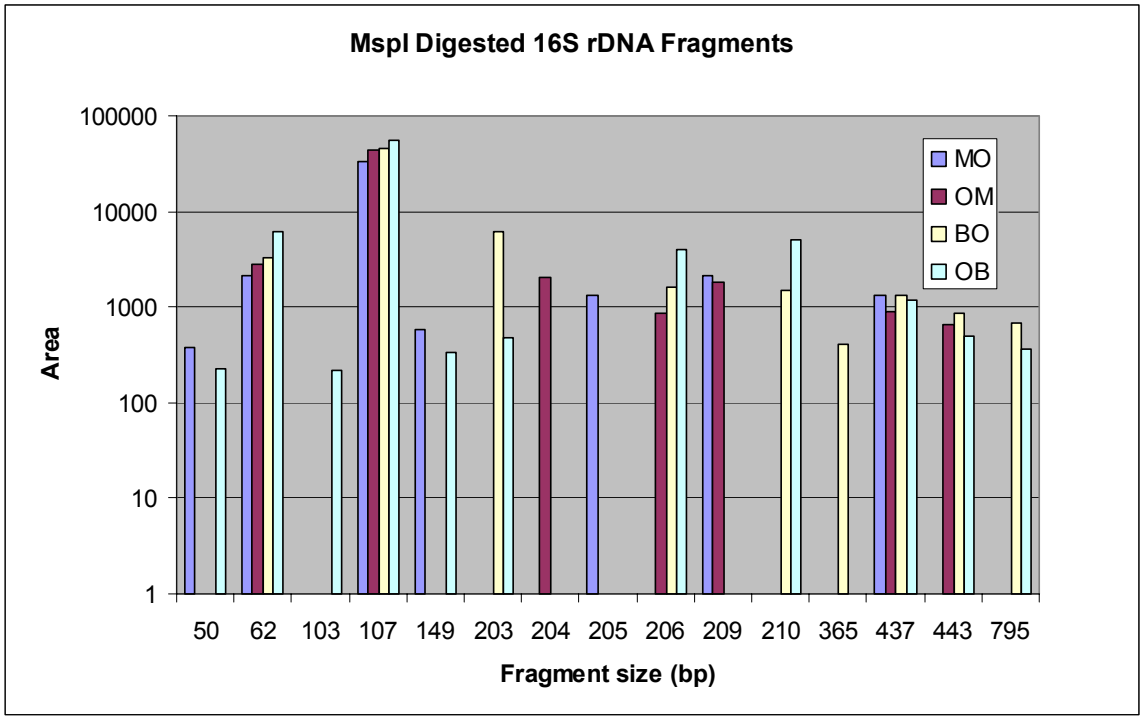
A.


RsaI Digested 16S rDNA Fragments
100000
10000
1000
100
10
1
Area
MO
OM
BO
OB
50 80 100 150 160 172 183 184 185 187 188 531 532 533 650 794 795 797
Fragment size (bp)

B.


HhaI Digested 16S rDNA Fragments
100000
10000
1000
100
10
1
Area
MO
OM
BO
OB
50 86 104 112 113 150 173 228 245 246 252 256 260 261 794
Fragment size (bp)

C.


MspI Digested 16S rDNA Fragments
100000
10000
1000
100
10
1
Area
MO
OM
BO
OB
50
62
103
107
149
203
204
205
206
209
210
365
437
443
795
Fragment size (bp)

APPENDIX

Table 1. Composition (% of total fatty acids) of the diets used in experiments outlined in Chapter 3.

	Control Diet		Intact Diet		Extract Diet	
%	DM	As Sampled	DM	As Sampled	DM	As Sampled
Dry Matter		59.17		60.75		59.10
Crude Protein	12.26	7.25	11.82	7.18	10.53	6.22
Unavailable Protein	1.80	1.06	2.04	1.24	2.09	1.23
Acid Detergent Fiber	29.35	17.36	30.74	18.67	31.42	18.57
Crude Fat	2.29	1.35	13.05	7.93	11.59	6.79
Calcium	0.96	0.57	0.58	0.35	0.57	0.34
Phosphorous	0.28	0.17	0.19	0.12	0.20	0.12
Sulfur	0.22	0.13	0.48	0.29	0.58	0.34
Magnesium	0.20	0.12	0.15	0.09	0.15	0.09
Sodium	0.70	0.41	0.76	0.46	0.90	0.53
Potassium	0.98	0.58	0.74	0.45	0.75	0.45
Copper, ppm	38.00	22.00	20.00	12.00	21.00	12.00
Iron, ppm	526.00	311.00	278.00	169.00	398.00	235.00
Manganese, ppm	145.00	86.00	88.00	53.00	87.00	51.00
Zinc, ppm	164.00	97.00	72.00	43.00	73.00	43.00
Net Energy, Mcal/lb	0.66	0.39	0.65	0.40	0.65	0.38

Table 2. LCFA (% of total FA) for all treatments at 0hr (A), 2hr (B), 4hr (C), 6hr (D), and 24hr (E) in batch cultures.

A. 0hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12	0.2[a]	0.4[b]	0.4[b]	0.05
i14	0.1[a]	0.0[b]	0.1[b]	0.02
C14:0	0.7[d]	8.8[e]	8.3[e]	0.91
i15	0.8[d]	0.3[e]	0.3[e]	0.05
a15	0.7[a]	0.2[b]	0.3[c]	0.03
C15:0	0.6	0.4	0.5	0.04
i16	12.1[d]	4.8[e]	3.9[e]	0.69
C17:0	0.3[d]	0.1[e]	0.2[e]	0.02
C20:0	0.4[d]	0.2[e]	0.2[e]	0.03
C18:3 C20:1	1.9[a]	0.7[b]	0.6[b]	0.25
C22:0	0.3	0.2	0.2	0.13

B. 2hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12	0.2[a]	0.7[b]	0.6[b]	0.09
i14	0.1	0.1	0.1	0.03
C14:0	0.6[d]	11.2[e]	10.5[e]	0.99
i15	1.4[a]	1.1[a]	0.6[b]	0.16
a15	0.6[d]	0.3[e]	0.3[e]	0.03
C15:0	0.5	0.6	0.5	0.05
i16	17.4[a]	10.3[b]	8.3[b]	2.37
C17:0	0.3[d]	0.1[e]	0.1[e]	0.02
C20:0	0.5	0.1	0.2	0.14
C18:3 C20:1	1.2[d]	0.5[e]	0.5[e]	0.06
C22:0	0.5[d]	0.2[e]	0.2[e]	0.02

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 2. (continued).

C. 4hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12	0.2[d]	0.5[e]	0.6[e]	0.05
i14	0.1	0.1	0.1	0.05
C14:0	0.6[d]	10.0[e]	11.8[e]	0.31
i15	1.2	1.0	0.5	0.23
a15	0.6[a]	0.3[b]	0.3[b]	0.07
C15:0	0.4	0.5	0.5	0.04
i16	17.8[a]	9.0[b]	11.3[b]	2.05
C17:0	0.3[d]	0.2[e]	0.1[e]	0.03
C20:0	0.3[d]	0.1[e]	0.1[e]	0.02
C18:3 C20:1	1.0[a]	0.4[b]	0.4[b]	0.12
C22:0	0.4[d]	0.2[e]	0.1[e]	0.05

D. 6hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12	0.2a	0.5b	0.5b	0.06
i14	0.1d	0.1d	0.1d	0.01
C14:0	0.6d	10.5e	10.4e	0.94
i15	1.1a	0.8a	0.3b	0.20
a15	0.7d	0.3e	0.3e	0.05
C15:0	0.5	0.5	0.5	0.05
i16	18.5d	8.6e	7.9e	1.56
C17:0	0.3d	0.2e	0.1e	0.02
C20:0	0.3d	0.2e	0.1e	0.02
C18:3 C20:1	0.9d	0.4e	0.3e	0.07
C22:0	0.5d	0.2e	0.2e	0.04

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 2. (continued).

E.	24hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12	0.4	0.5	0.4	0.09
i14	0.4[d]	0.2[e]	0.2[e]	0.02
C14:0	1.3[d]	9.1[e]	9.7[e]	0.58
i15	3.3[a]	1.6[b]	1.4[b]	0.48
a15	1.3[d]	0.6[e]	0.5[e]	0.09
C15:0	1.1[d]	0.6[e]	0.7[e]	0.05
i16	24.8[d]	10.5[e]	11.7[e]	1.86
C17:0	0.4[d]	0.2[e]	0.1[e]	0.02
C20:0	0.3	0.1	0.2	0.05
C18:3 C20:1	0.5[d]	0.2[e]	0.1[e]	0.07
C22:0	0.5[d]	0.2[e]	0.2[e]	0.03

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 3. Amount (mg) of LCFA for all treatments at 0hr (A), 2hr (B), 4hr (C), 6hr (D), and 24hr (E) in batch cultures.

A. 0hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12	0.1	0.3	0.3	0.09
i14	0.0	0.0	0.1	0.01
C14:0	0.2[a]	5.6[b]	6.7[b]	1.52
i15	0.2	0.2	0.3	0.05
a15	0.2	0.1	0.2	0.05
C15:0	0.1[a]	0.3[a]	0.4[b]	0.07
i16	2.7	3.1	3.3	0.56
C17:0	0.1	0.1	0.1	0.02
C20:0	0.1	0.1	0.1	0.01
C18:3 C20:1	0.4	0.4	0.5	0.09
C22:0	0.1	0.1	0.1	0.36
Total Lipid	34.7	86.7	94.0	

B. 2hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12:0	0.1[e]	0.5[f]	0.4[f]	0.07
i14	0.0	0.1	0.0	0.0
C14:0	0.1[d]	7.8[e]	7.5[e]	0.90
i15	0.3[d]	0.8[e]	0.4[d]	0.07
a15	0.1[a]	0.2[b]	0.2[b]	0.02
C15:0	0.1[d]	0.4[e]	0.4[e]	0.03
i16	3.7	7.2	6.0	1.11
C17:0	0.1[a]	0.1[b]	0.1[b]	0.01
C20:0	0.1	0.1	0.1	0.02
C18:3 C20:1	0.3	0.3	0.4	0.03
C22:0	0.1	0.1	0.1	0.12
Total Lipid	47.0	78.7	84.3	

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 3. (continued).

C. 4hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12:0	0.1[e]	0.4[f]	0.5[f]	0.04
i14	0.0	0.1	0.1	0.01
C14:0	0.2[e]	7.2[f]	9.3[f]	0.78
i15	0.3	0.7	0.4	0.14
a15	0.2[a]	0.2[b]	0.3[b]	0.23
C15:0	0.1[d]	0.4[e]	0.4[e]	0.04
i16	4.4	6.4	8.8	1.34
C17:0	0.1[d]	0.1[e]	0.1[e]	0.01
C20:0	0.08	0.10	0.10	0.005
C18:3 C20:1	0.3	0.3	0.3	0.06
C22:0	0.1	0.1	0.1	0.04
Total Lipid	42.0	88.3	102.7	

D. 6hr

Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12:0	0.0e	0.4f	0.4f	0.02
i14	0.0	0.0	0.1	0.01
C14:0	0.0d	7.5e	8.5e	0.67
i15	0.4	0.6	0.2	0.10
a15	0.2	0.2	0.2	0.04
C15:0	0.1d	0.4e	0.4e	0.03
i16	4.6	6.2	6.5	0.73
C17:0	0.1a	0.1b	0.1b	0.01
C20:0	0.1	0.1	0.1	0.01
C18:3 C20:1	0.2	0.3	0.3	0.04
C22:0	0.1	0.1	0.2	0.02
Total Lipid	47.0	77.3	110.7	

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 3. (continued).

E.	24hr			
Fatty Acid	Control (C)	Extract (E)	Intact (I)	SE
C12:0	0.1[a]	0.4[b]	0.3[b]	0.05
i14	0.1	0.2	0.2	0.02
C14:0	0.4[e]	7.4[e]	7.8[e]	0.70
i15	1.1	1.3	1.1	0.32
a15	0.4	0.5	0.4	0.07
C15:0	0.4	0.5	0.5	0.06
i16	8.1	8.5	9.4	1.70
C17:0	0.1	0.1	0.1	0.02
C20:0	0.1	0.1	0.1	0.06
C18:3 C20:1	0.2[a]	0.2[a]	0.1[b]	0.02
C22:0	0.2	0.2	0.2	0.03
Total Lipid	76.0	92.7	111.0	

[a,b,c] Means with different superscripts differ $p<0.05$
[d,e,f] Means with different superscripts differ $p<0.01$

Table 4. Microbial populations of all fermentors sorted by unique species per order.

Order	C d4	C d10	C d16	M	B	O	MO	BO	OM	OB
Acidobacteriales	1				1		1	1		
Actinomycetales	6	8	2	3	11	6	1	6	3	6
Aeromonadales	1			3	4	3.5			2	
Alteromonadales	0.5	1	1	2	5	1				2
Bacillales	6.5	4	1	7	12	5.5	6	1	3	11
Bacteroidales	1.5	3	1	2	1	1		1	1	1
Bifidobacteriales					1					
Burkholderiales	4.5	5	1	4	14	7	3	6	5	7
Campylobacterales	1.5	3	1		2	1.5		3		2
Chlorobiales								1		
Chloroflexales				1					1	
Chromatiales					1					1
Chroococcales					1			1		
Clostridiales	2		1	1	5	1.5	5		2	5
Corynebacterineae	1	3	3		3	0.5		2		
Desulfovibrionales	1.5			2	2				2	
Desulfuromonadales										1
Enterobacteriales				2						
Entomoplasmatales		1			2			1		4
Flavobacteriales	1					0.5		1		
Hydrogenophilales					1					
Lactobacillales	1	1	1		1	1	2	1	1	1
Methylophilales					1					
Micrococcineae		1			3	0.5	1			
Multiple	78	82	39	45	177	59	21	54	57	89
Mycoplasmatales		1			2					5
Myxococcales	0.5									
Neisseriales										1
Nitrosomonadales	0.5			1	1	0.5		1		
Nitrospirales	0.5				1	0.5				
Oceanospirillales	2.5	1	4	2	12	3.5		3	1	6
Pasteurellales		1			1	0.5				
Planctomycetales	0.5									1
Pseudomonadales	2	1	1	2	12	1		1	2	4
Rhizobiales					1					
Rhodobacterales					1					
Rhodocyclales					1	0.5				
Rhodospirillales					1					
Sphingobacteriales	2	2		1	4	1.5		1	2	1
Streptomycineae				2		0.5				
Syntrophobacterales			1		1		1	1	1	2
Thermoanaerobacteriales						1				2
Unclassified	163.5	146	66	97	317	136	47	108	131	191
Verrucomicrobiales	1.5				2					
Vibrionales					3			3	1	
Xanthomonadales	0.5		2		4				1	3
Total	**281**	**264**	**125**	**177**	**612**	**234**	**88**	**197**	**216**	**346**

Table 5. Microbial populations of all fermentors sorted by unique species per family.

Family	C d4	C d10	C d16	M	B	O	MO	BO	OM	OB
Acidaminococcaceae							1		1	1
Acidobacteriaceae	1				1					
Actinomycetaceae					1					2
Aeromonadaceae	1			3	4	3.5			2	
Alcaligenaceae	0.5	2		1	3	3.5	1	1	2	2
Alcanivoracaceae	1	1	3		4	1		2		3
Alicyclobacillaceae		1			1				1	1
Alteromonadaceae			1		3	0.5				1
Bacillaceae	5.5	3	1	4	6	5	4		2	9
Bacteroidaceae	1	2		1		0.5		1	1	1
Beutenbergiaceae								1		
Bifidobacteriaceae					1					
Brevibacteriaceae	1	2	1		2	1.5	1	1		2
Burkholderiaceae	2	2	1	1	5	3		2	2	3
Campylobacteraceae	1.5	3	1		2	1.5		3		2
Cellulomonadaceae	1.5	1		1	1	1	1		1	1
Chlorobiaceae								1		
Chloroflexaceae				1					1	
Chromatiaceae										1
Clostridiaceae							1			
Comamonadaceae	0.5	1			3		1	1	1	
Corynebacteriaceae	0.5	1				0.5		1		
Desulfovibrionaceae	1.5			2	2				2	
Ectothiorhodospiraceae					1					
Enterobacteriaceae				2						
Eubacteriaceae	1								1	1
Flavobacteriaceae	1					0.5		1		
Flexibacteraceae	1	2		1	4	1.5		1	2	1
Gallionellaceae				1				1		
Geobacteraceae										1
Gordoniaceae	0.5									
Halomonadaceae	1			1	5	2.5			1	1
Hydrogenophilaceae					1					
Lachnospiraceae			1		3	1.5	1			1
Leuconostocaceae	1	1	1		1	1	1	1	1	1
Listeriaceae				2	1		1			
Methylocystaceae					1					
Methylophilaceae					1					
Microbacteriaceae					2					
Micrococcaceae	1	1			1	1		1		
Moraxellaceae		1	1		2			1		
Moritellaceae		1		1	1					
Multiple	80.5	82	39	47	181	59	23	56	57	91
Mycobacteriaceae	0.5	3	3		5	0.5		4		
Mycoplasmataceae		1			2					5
Neisseriaceae										1

Table 5. (continued).

Family	C d4	C d10	C d16	M	B	O	MO	BO	OM	OB
Nitrosomonadaceae	0.5				1	0.5				
Nocardiaceae	0.5	2	1		2	0.5				
Oceanospirillaceae			1	1	2			1		2
Oxalobacteraceae					1					
Paenibacillaceae					3	0.5				1
Pasteurellaceae		1			1	0.5				
Pseudoalteromonadaceae	0.5				1					1
Pseudomonadaceae	2			2	10	1			2	4
Rhodobacteraceae					1					
Rhodocyclaceae					1	0.5				
Rhodospirillaceae					1					
Rikenellaceae	0.5	1	1	1	1	0.5				
Shewanellaceae				1		0.5				
Sphingobacteriaceae	0.5									
Spiroplasmataceae		1			2			1		4
Staphylococcaceae	1			1	1		1	1		
Streptococcaceae							1			
Streptomycetaceae	1.5	2		4	2	2.5			2	1
Syntrophobacteraceae			1		1		1	1	1	2
Syntrophomonadaceae					1					
Thermoanaerobacteriaceae						1				2
Unclassified	166	146	66	98	320	137	49	110	131	194
Verrucomicrobiaceae	1.5				2					
Vibrionaceae					3			3	1	
Xanthomonadaceae	0.5		2		4				1	3
Total	**281**	**264**	**125**	**177**	**612**	**234**	**88**	**197**	**216**	**346**

Table 6. Microbial populations of all fermentors sorted by unique species per genus.

Genus	C d4	C d10	C d16	M	B	O	MO	BO	OM	OB
Acinetobacter					1					
Actinobacillus		1			1	0.5				
Actinomyces					1					2
Aeromonas	1			2	3	3			2	
Alcaligenes		1		1	2	2			1	2
Alcanivorax	1	1	3		4	1		2		3
Alicyclobacillus		1			1					1
Alkaliflexus	0.5	1	1	1	1	0.5				
Alkalilimnicola					1					
Alkanindiges		1	1		1			1		
Arcobacter	0.5	1			1	0.5		1		1
Arthrobacter	0.5				1					
Bacillus	5	3	1	3	6	5	4		2	7
Bacteroides	1	2		1		0.5		1	1	1
Bifidobacterium					1					
Bordetella		1			1			1		
Brevibacterium	1	2	1		2	1.5	1	1		2
Buchnera				1						
Burkholderia		1	1		1	1		1		1
Campylobacter	0.5	1			1	0.5		1		
Capnocytophaga	1									
Catenibacterium					2					1
Cellulomonas	1.5	1		1	1	1	1		1	1
Cellvibrio									1	1
Chlorobium								1		
Chromobacterium										1
Citrobacter				1						
Clostridium							1			
Comamonas								1		
Corynebacterium	0.5	1				0.5		1		
Cytophaga	0.5	1		1	2	0.5		1	1	1
Dechloromonas					1					
Delftia					1					
Desulfovibrio	0.5			1	1				1	
Enhygromyxa	0.5									
Eubacterium	1									1
Flavobacterium						0.5		1		
Frateuria			1		1					1
Gallionella				1				1		
Georgenia								1		
Geothermobacter										1
Gordonia	0.5									
Halobacillus				1						1
Halochromatium										1
Halomonas	1			1	4	2.5			1	
Herbaspirillum					1					
Hymenobacter	0.5				1					
Kitasatospora	0.5	1		3	1	1.5			1	

Table 6. (continued)

Genus	C d4	C d10	C d16	M	B	O	MO	BO	OM	OB
Leuconostoc	1	1	1		1	1	1	1	1	1
Listeria				2	1		1			
Macrococcus				1						
Marinobacter			1		3					1
Marinomonas			1	1	2			1		2
Megasphaera							1			
Methylobacillus					1					
Methylocystis					1					
Microbacterium					1					
Moritella		1		1	1					
Multiple	83.5	84	40	48	188	61	23	58	60	94
Mycobacterium	0.5	3	3		5	0.5		4		
Mycoplasma		1			2					5
Natronoanaerobium	0.5			1	1		1			1
Nitrosomonas	0.5				1	0.5				
Oceanimonas				1	1	0.5				
Paenibacillus					2	0.5				1
Pasteuria									1	
Pseudoalteromonas	0.5				1					1
Pseudomonas	2			2	10	1			1	3
Pseudoxanthomonas										1
Pusillimonas						1			1	
Ralstonia	2	1		1	4	2		1	2	2
Rhodococcus	0.5	2	1		2	0.5				
Rhodothermus	0.5									
Ruminococcus			1		1	1.5	1			
Salinicoccus	0.5				1		1	1		
Selenomonas									1	1
Shewanella				1		0.5				
Sphingobacterium	0.5									
Spiroplasma		1			2			1		4
Stenotrophomonas			1		1					
Streptococcus							1			
Streptomyces	1	1		1	1	1			1	1
Synechococcus					1			1		
Syntrophobacter			1		1		1	1	1	1
Syntrophomonas					1					
Taylorella	0.5					0.5	1			
Thauera						0.5				
Thermovenabulum						1				2
Thiobacillus					1					
Tistrella					1					
Unclassified	167.5	148	66	98	323	138	49	110	133	194
Xanthomonas	0.5				2				1	
Xylella										1
Vibrio					2			2	1	
Total	**271**	**258**	**123**	**170**	**591**	**225**	**81**	**194**	**209**	**330**

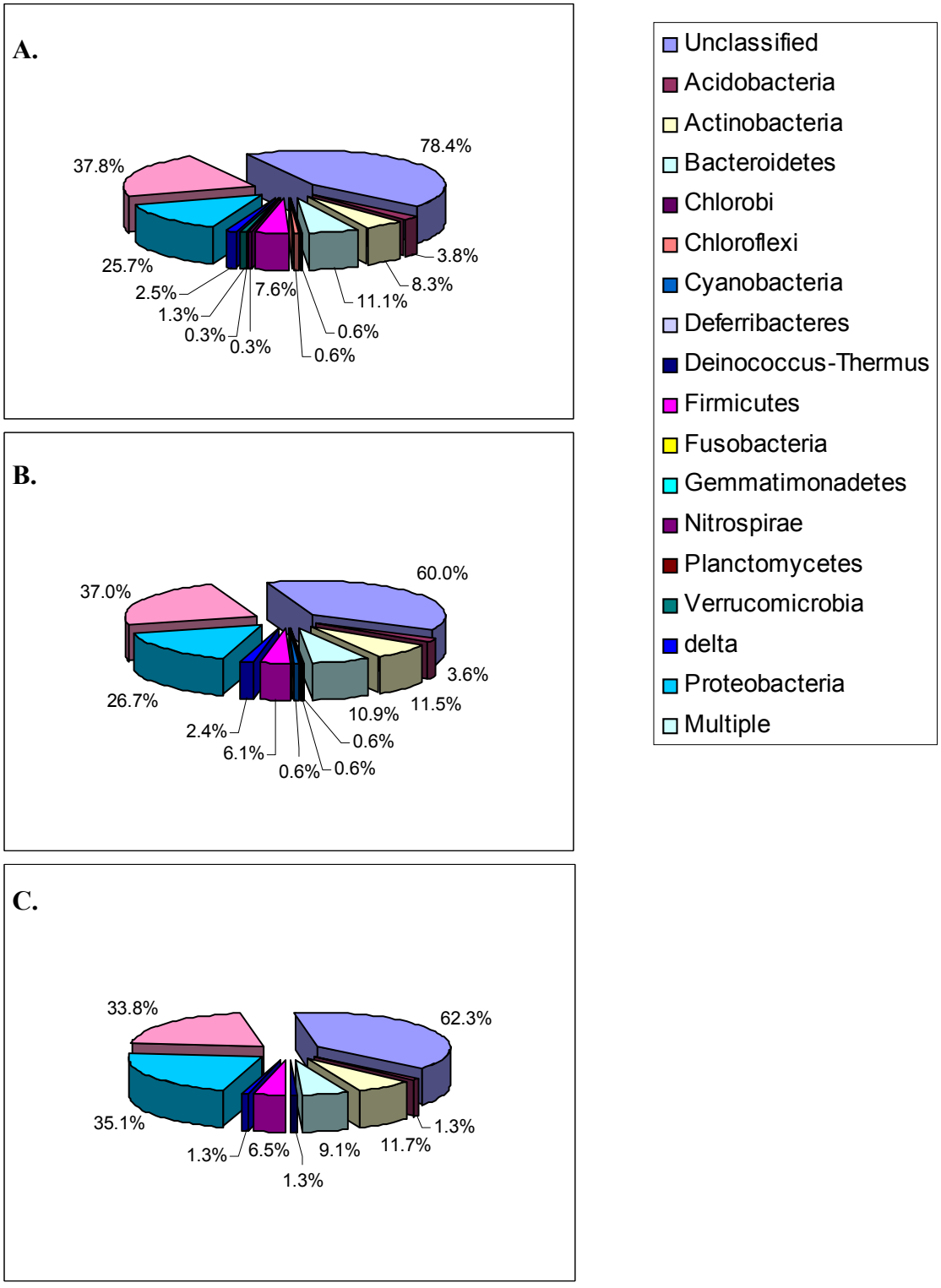


Figure 1. Graphical representation of the percentage of unique species identified per phylum of control on day 4 (A), 10 (B), and 16 (C).

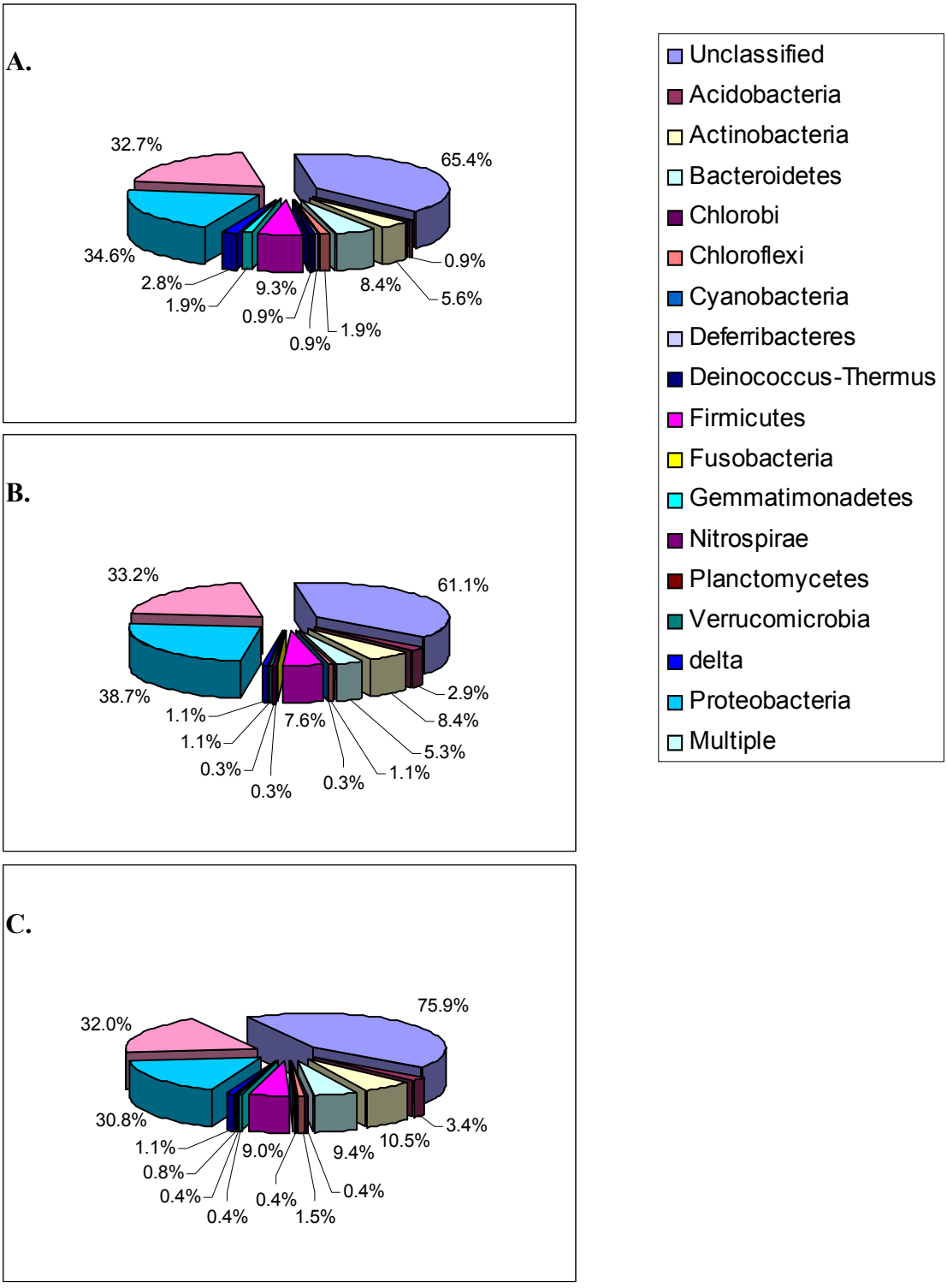


Figure 2. Graphical representation of the percentage of unique species identified per phylum of additive effects monensin (A), bacitracin (B), and oil (C).

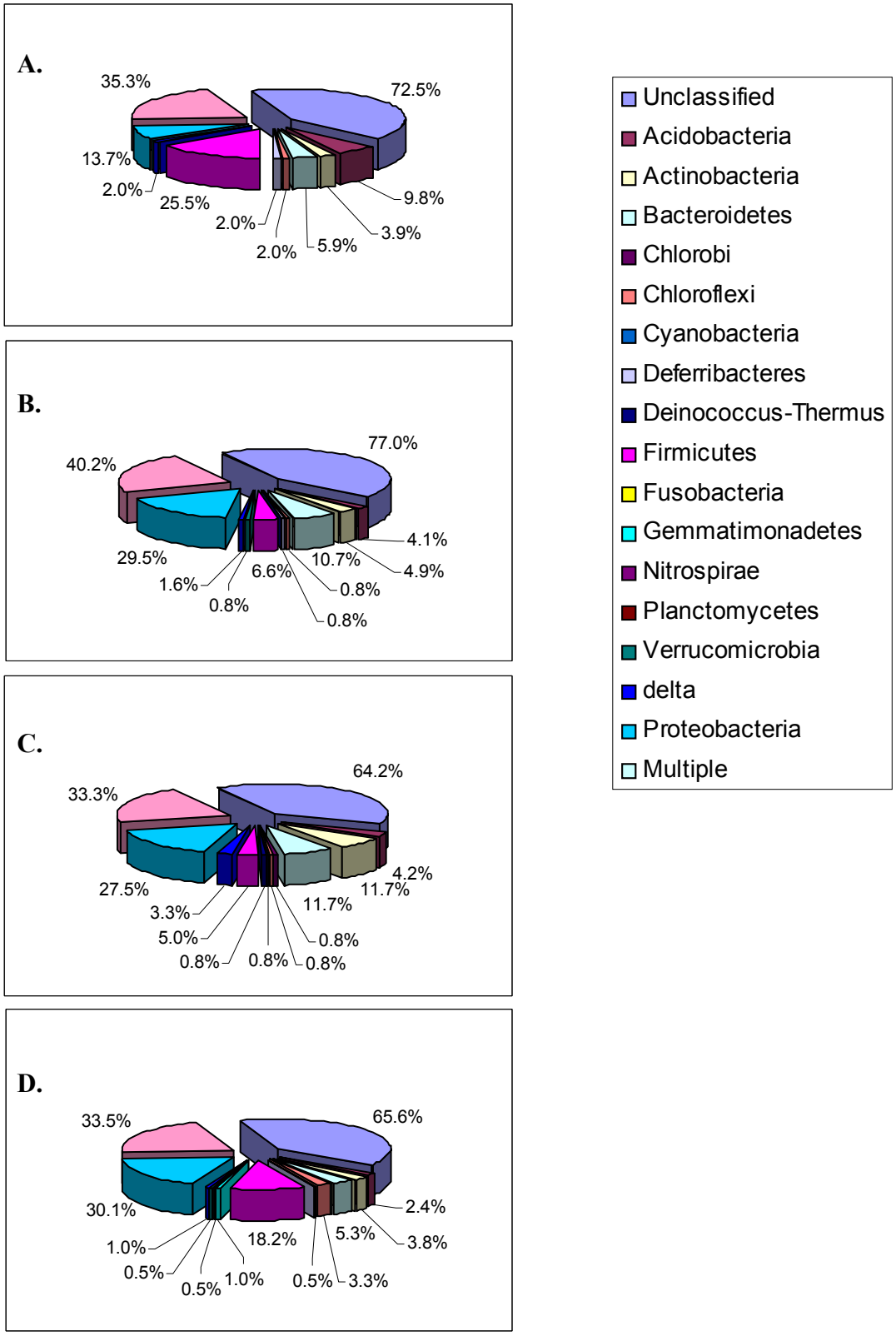


Figure 3. Graphical representation of the percentage of unique species identified per phylum of sequence effects MO (A), OM (B), BO (C), and OB (D).