Rumen microbial community analysis and methanogenesis during adaptation to monensin

by

Jenelle Bouchard

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment to the requirements of

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Of

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ABSTRACT

The aim of this study was to characterize the adaptation of rumen microbial populations and methane emissions to ionophores when fed to cattle over a 12 wk period. Methane emissions from ruminants contribute approximately 90% of the greenhouse gas emissions from animal agriculture (Kebreab et al. 2006). The gram-positive specific ionophore antibiotic, monensin, may lower methane production in the rumen, however the effects appear to be short term (weeks) and there is a rebound effect in which emissions return to baseline levels. We hypothesized that gram-positive bacteria, but not methanogenic archaea, adapt to monensin and that the methane emission rebound can largely be explained by adaptation of gram-positive hydrogen producing bacteria. Rumen samples were taken weekly for 12 wk from cattle fed either a high grain or a high forage diet with or without monensin (Guan et al. 2006). Methane emissions were simultaneously measured. Microbial populations were characterized using terminal restriction fragment length polymorphisms (TRFLP) analysis. Key microbial species were quantified with real-time PCR (RT-PCR). TRFLP indicated that at the phylum level community structure was relatively stable showing no statistical difference ($P < 0.05$) between treatment or diet as well as no difference over time. There was a noticeable prevalence of members of the phylum Verrucomicrobia. No members of this phylum have been cultured from the rumen but may be involved in polysaccharide degradation or anaerobic methane oxidation. RT-PCR indicated that adaptation occurred partly within the hydrogen producing gram-positive bacteria on the concentrate diet and the effects on methanogens were strongly correlated with these population shifts. In the

forage diet the adaptation to ionophores also occurred but modulation of gram-positive bacteria appeared to be much more subtle. The methanogenic archaea were not directly affected by monensin, the observed abundance changes were related to the decrease in the abundance of ciliate protozoa and the available hydrogen in the rumen. Methane emissions returned to baseline levels two wk earlier in grain than forage diets and this is likely the result of higher turnover rates in grain based rations. A low correlation between methane levels and temperature showed that the influence of temperature on the data was not large but could not exclude it.

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Finally, I would like to thank my family. Thank you to my parents and siblings for your love, guidance and unconditional support. Thank you also to all my extended family for your encouragement. To the Hamblins, thank you for your love, advice and for making these last few years very special. Last by certainly not least, I would like to thank my husband Kevin, thank you for your love and strength, and thank you for always believing in me.

DEDICATION

I dedicate this work to my parents. Dad, it was you that stimulated my love of animals and agriculture. Mom, you instilled in me the importance and value of an education. You both have taught me to work hard and never give up on my dreams. You both have given so much, and whenever I needed you, you were there.

FOREWORD

A part of this thesis has been written in manuscript format. This thesis is organized with an abstract of the thesis, a general introduction and a literature review before the manuscript, which is followed by a general discussion and conclusions. The format used to write this thesis is that of the Canadian Journal of Animal Science. The authors and title of the manuscript is:

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ABBREVIATIONS

ATP	Adenosine Triphosphate
CH_4	Methane
CO_2	Carbon Dioxide
CT	Threshold Cycle
H_2	Hydrogen
MICA	Microbial Community Analysis
NAD+	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotimamide Adenine Dinucleotide (reduced form)
PAT	Phylogenetic Assignment Tool
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project
RT-PCR	Real Time Polymerase Chain Reaction
TRF	Terminal Restriction Fragment
TRFLP	Terminal Restriction Fragment Length Polymorphism
VFA	Volatile Fatty Acid

1.0 GENERAL INTRODUCTION

In animal agriculture, ruminant livestock contribute the majority of greenhouse gas emissions with methane being the predominant pollutant (Kebreab et al. 2006). Methane is a byproduct of rumen fermentation and is eructated as waste from the animal into the atmosphere (Moss et al. 2000). The global pressures to reduce greenhouse gas emissions have made the need for a long-term strategy to mitigate emissions from ruminants even more pressing. Research focusing on methane depression in ruminants has explored many possible sources including plant compounds such as tannins (Woodward et al. 2001) and essential oils (Beauchemin and McGinn 2006). Vaccines against rumen methanogens (Wright et al. 2004) have been suggested but the feeding of ionophores is very appealing because of its ease of use in feed formulation and effectiveness in reducing enteric methane (Guan et al. 2006).

Ionophores are lipophilic molecules that permeate the cell membrane of bacteria, protozoa, fungi, and even higher organisms (Pressman 1976). They act by dissipating ion gradients leading to an ionic overload in the cytoplasm of a cell (Russell and Strobel 1989). To combat this overload, the cell activates ion pumps to drive ions out, which leads to the depletion of adenosine triphosphate (ATP) energy. If the ion imbalance is large, the cells exhaust all of their ATP which leads to cell death (Russell and Strobel 1989; Callaway et al. 2003). Ionophores are predominately affective against gram-positive bacteria and this is due to the nature of their cell wall (Russell and Strobel 1989). Gram-positive bacteria have a single thick permeable peptidoglycan layer resting above the phospholipid bilayer that is the cell membrane (Callaway et al. 2003). The cell wall

of gram-negative bacteria differs in that it has two thin layers of peptidoglycan as well as lipopolysaccharides. These lipopolysaccharides make the insertion of ionophores into the cell membrane difficult (Russell and Houlihan 2003).

Many ruminal gram-positive bacteria are also hydrogen producers, and as hydrogen is required for methane formation these species form an important link between mode of action of ionophores and methane generation (Russell and Rychlik 2001). Bacteria produce hydrogen during the production of volatile fatty acids such as acetate (Russell and Rychlik 2001), therefore if the gram-positive, hydrogen producing bacteria are depleted there is little hydrogen available for methanogenesis. A previous study performed at the University of Manitoba by Guan et al. (2006) demonstrated that when monensin was supplemented to feedlot steers on either forage or grain diets methane levels decreased significantly, however it was only short term and methane levels returned to baseline after several weeks. This suggests possibility that the gram-positive rumen bacteria may adapt to monensin.

The purpose of this thesis research was to evaluate the microbial adaptation to monensin in a microbial community context. Rumen microbial populations can now be studied without the need for cultivations, using molecular techniques such as the polymerase chain reaction (PCR), terminal restriction fragment length polymorphism (TRFLP) and real time PCR (RT-PCR). Using these techniques rumen fluid from the Guan et al. (2006) study were analyzed to determine which populations in the rumen are the most affected by monensin, and which ones are capable of adaptation.

2.0 LITERATURE REVIEW

2.1 The Rumen

2.1.1 Rumen Microbes

The rumen is a complex ecosystem that has been extensively researched to attain a more complete mechanistic understanding. The rumen consists of billions of microbial species which are predominately anaerobic. These include bacteria, protozoa, fungi, many of which are continually being identified with the use of molecular techniques (Russell and Rychlik 2001). With more than 200 species isolated, it has been estimated that bacterial counts in the rumen are as high as 10^{10} cells/mL of rumen fluid or 10,000,000,000 bacteria/mL (Callaway 2005). The bacteria are the best understood component of the ruminal ecosystem and there is much less known about the protozoa and even less about the fungi.

The microorganisms in the rumen are extremely diverse with each serving a unique purpose in the ecosystem. The bacteria are primarily involved with fermentation in the rumen as they can break down nearly all ingested feedstuffs (Callaway 2005). The protozoa, which also ferment dietary components and are involved in ruminal nitrogen cycling, are major consumers of rumen bacteria therefore acting as their regulators (Callaway 2005). All the microbial populations in the rumen live in a symbiotic relationship with the animal; the rumen provides a habitat and nutrition to the microorganisms while the microorganisms provide necessary nutrients to the animal such as volatile fatty acids (VFA) for energy, microbial biomass for protein, and vitamins

(Callaway 2005; Russell and Rychlik 2001). The bacteria also play a vital role in physiological and immunological functions (Mackie and Cann 2005).

The microorganisms digest the consumed feedstuffs and provide nutrients to the animal through fermentation. The primary diet of ruminants is high in forages, which possess structural carbohydrates such as cellulose and hemicellulose. Ruminants are unable to break down these plants compounds as they cannot produce the required digestive enzymes, cellulase and hemicellulase. Rumen bacteria, however, do produce these enzymes, and are capable of breaking down the forages releasing glucose which provides nutritional value to the animal (Russell and Rychlik 2001; Prescott et al. 2002). Rumen bacteria can also ferment starches and sugars found in high energy grain diets into VFA for energy, this is of major importance since grain diets have become much more common for domestic ruminants to increase meat and milk production (Russell and Rychlik 2001).

The rumen provides ideal living conditions for microorganisms. Primarily, the rumen is anaerobic or absent of oxygen and some rumen microorganims are so sensitive to oxygen that they cannot tolerate even the slightest exposure (Prescott et al. 2002). The rumen pH is normally between 6.0 and 6.9 with an optimum temperature at 39 °C. The rumen is a very unique ecosystem, providing a continuous source of nutrients and growth making it truly symbiotic.

2.1.2 Rumen Fermentation

Fermentation is an anaerobic process which degrades substrates into usable products. In the rumen, microorganisms utilize consumed carbohydrate feedstuffs and ferment them through a series of oxidation/reduction reactions into volatile fatty acids

(VFA) for energy, and carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2) as by-products (Figure 1) (Callaway 2005; Chiba 2007; Russell and Rychlik 2001). During fermentation, carbohydrates must first be oxidized into pyruvate through the glycolysis pathway which utilizes the reducing equivalent nicotinamide adenine dinucleotide (NAD^+-oxidized or NADH-reduced forms). During glycolysis, NAD^+ is reduced to NADH and in order for fermentation to continue NAD^+ must be regenerated, which occurs mainly through the reduction of CO_2 to CH_4 (Prescott et al. 2002; McAllister and Newbold 2008). Pyruvate is than converted into one of the major VFAs.

The major VFAs produced in the rumen are acetate, butyrate and propionate which are the major source of carbohydrate and energy for the animal (Callaway 2005; Cheeke 2005). Microbial cells are the main source of high quality protein for the animal especially for the essential amino acids (Ishler et al. 1996). These amino acids are absorbed in the small intestine and used for animal growth and meat or milk production (Ishler et al. 1996). Many of the vitamin requirements for the ruminant are also met by fermentation, all of the water soluble vitamins as well as vitamin K are all synthesized leaving only vitamins A, D and E to be supplemented (Cheeke 2005). Fermentation is efficient as it allows ruminants to consume inexpensive low quality feed and still meet all their nutrient requirements.

Not all products of fermentation are utilized by the animal and in some cases their production represents a loss of feed efficiency and is detrimental to the environment. Carbon dioxide and methane gases are by-products of fermentation, and an accumulation of these gases causes bloat in the rumen, therefore they need to be released by a process called eructation or belching (Cheeke 2005). The release of these greenhouse gases

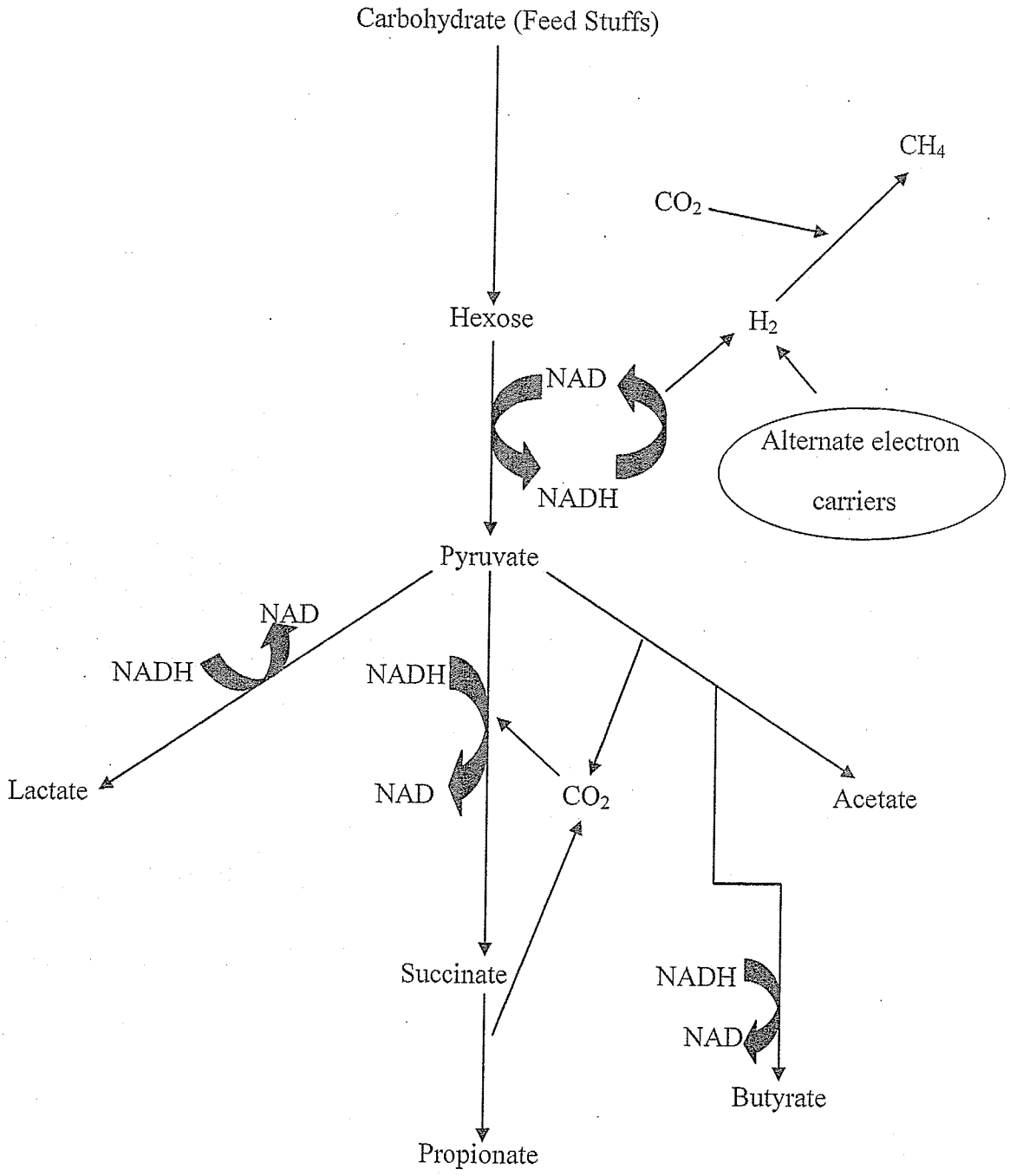

Figure 2.1: The major biochemical pathways of carbohydrate fermentation in the rumen

Modified from Russell and Rychlik 2001

into the atmosphere is harmful to the environment and is what ruminant research is attempting to inhibit. Another environmental issue is the excretion of ammonia in urine building up in soil causing nitrogen pollution. Not all ammonia produced in fermentation is used by the animal and therefore it is classified as a waste product to the animal and is released through urination (Callaway 2005). As well the production of carbon dioxide, methane and ammonia represents an energy loss for the animal as they serve no nutritive purpose, for example 1 gram of produced methane represents a feed energy loss of 55.2 kJ (Wittenberg 2003). To combat these losses in feed efficiency and harmful impacts on the environment, strategies to reduce methane production have taken a priority in ruminant research and to date the one that has seen the most promise is the use of ionophores such as monensin in the diet (Guan et al. 2006).

2.1.3 Hydrogen Production and Utilization

Hydrogen gas (H_2) is produced during fermentation as a byproduct en route to the production of acetate, the primary VFA produced in the rumen (Russell and Rychlik 2001). Although acetate is the primary VFA, the concentration of hydrogen in the rumen is typically low, as it is promptly used in downstream reactions (Hungate 1966). Hydrogen is produced by microorganisms using hydrogenase enzymes which take electrons from reduced cofactors such as NADH that are also produced during fermentation reactions, this reaction is very thermodynamically unfavorable and will only chemically proceed if the product is quickly consumed in other reactions (Russell and Rychlik 2001). Being that methanogens require hydrogen to produce methane, they provide an outlet for hydrogen disposal (Russell and Rychlik 2001). Another sink for H_2 in the rumen are dicarboxylic acids such as sodium fumarate and sodium acrylate, which

are precursors in the production of propionate (McAllister and Newbold 2008; Newbold et al. 2005). An *in vitro* study by Newbold et al. (2005) showed both fumarate and acrylate are effective hydrogen sinks to remove electrons from methanogenesis. Fumarate and acrylate captured 44% and 22% respectively of the hydrogen previously used for methane production (Newbold et al. 2005). It is important the H_2 is quickly removed from the rumen environment as its build up would prevent further fermentation of feedstuffs (Weimer 1998). Since hydrogen plays such a vital role in the production of methane this may be a good area to target for decreasing methane emissions in livestock.

2.2 Rumen Methanogens & Methanogenesis

2.2.1 Methanogen Populations

Although methanogens make up only a small portion of rumen microbial populations, contributing 0.3 – 3.0% of the 16S and 18S rRNA (Yanagita et al. 2000), they play a very significant role. Methanogens are highly anaerobic, fastidious organisms, and are distinctive from other rumen organisms as they all produce methane as a catabolic end product (Bergey 1994). Methanogens are members of the phylum Euryarchaeota in the domain Archaea with 28 genera and 113 species of methanogens classified to date (Garrity 2007). Within the rumen, only seven species in five genera are known to reside and include *Methanobacterium formicicum*, *M. bryantii*, *Methanobrevibacter ruminantium*, *M. millerae*, *Methanomicrobium mobile*, *M. olleyae*, and *Methanoculleus olentangyi* with the majority (61.6%) found in the genus *Methanobrevibacter* (Janssen and Kirs 2008). It has also been reported that *Methanosarcina spp.* have been cultured from the rumen (Jarvis et al. 2000), however

some researchers do not believe them to be a main part of the rumen archaeal community (Janssen and Kirs 2008). The role of the methanogens in the rumen is to keep fermentation cycling efficiently, to do this they consume the hydrogen that is produced during fermentation reactions to produce methane, which results in a continuous fermentation rate as well as a nutritionally more favorable sequence of VFA production by rumen microorganisms (Kamra 2005; McAllister and Newbold 2008).

The methanogenic species named above grow in the rumen using H_2 and to a lesser extent formate as energy sources, utilizing the electrons from the hydrogen or formate to convert carbon dioxide to methane (Janssen and Kirs 2008), this is the process of methanogenesis. Other compounds have also been identified as substrates for methanogenesis; Jarvis et al. (2000) isolated a strain of *Methanosarcina barkeri* from grazing cattle that used CO_2/H_2, acetate, and/or methyl containing compounds to convert carbon dioxide to methane. The use of substrate is based completely on whether or not the methanogen possesses cytochromes. Methanogens that possess cytochromes, those of the order *Methanosarcinales*, are able to use methanol, methylamines, acetate and/or CO_2/H_2 as substrates for methanogenesis (Ohene-Adjei et al. 2007; Gottschalk 1988). While the methanogens without cytochromes are those that utilize only CO_2 and H_2 and/or formate for methanogenesis, these belong to the orders *Methanobacteriales, Methanococcales, Methanomicrobiales,* and *Methanopyrales* (Ohene-Adjei et al. 2007).

Methanogens are found in the rumen as planktonic organisms as well as symbionts with protozoa. They are found in different fractions of the rumen, and grow at different rates but they all perform methanogenesis (Janssen and Kirs 2008). Those methanogens associated with protozoa can either be attached to their surface or live

within the cells as endosymbionts (Christophersen et al. 2004); and the majority have been identified as relatives of *M. smithii* and *M. ruminantum* species (Tokura et al. 1999) and to a lesser extent the genus *Methanomicrobium* (Janssen and Kirs 2008). In 1994, Finlay et al. found that on the surface of rumen ciliate protozoa most commonly less than 10 and never more than 20 methanogenic organisms could exist, as well it was seen using electron microscopy those methanogens living inside the protozoa. The symbiotic link between methanogens and protozoa brought about another theory for decreasing methane production in the rumen, which was to target the protozoa populations for death and in turn affect the associated methanogens. Defaunation is the process which eliminates protozoa from the rumen, and it has been shown to reduce methane production by 30 – 45% (Tokura et al. 1999).

Rumen methanogens have been very difficult to quantify as they are extremely sensitive to oxygen (Zinder 1998). Using pure culture methods the highest level of care must be employed to avoid any contact with oxygen because methanogen growth is stunted when as little as 0.8 μL of oxygen is present and completely inhibited when levels reach 6 μL (Hungate 1966). Culture techniques have advanced over time to study methanogens resulting in the introduction of anaerobic glove box cabinets. The cabinets commonly used today are devoid of oxygen providing the ideal environment for anaerobic study, and researchers are now able to dispense media and plate out cells with confidence (Zinder 1998). The other dependable culture method used by laboratories that are without anaerobic cabinets includes the use of special air tight tubes which are pressurized with H_2 and CO_2 gases as growth substrates, this method also provides efficient growth of methanogens (Zinder 1998).

It was not until the development of molecular methods that rumen methanogens could be better studied, and even still the phylogeneticity of the rumen archaea are not completely understood. This was shown by Whitford et al. in 2001, when a phylogenetic study was performed in an attempt to identify rumen methanogens using the archaeal 16S rRNA gene. They found that the gene did not encompass all the phylogenetic diversity of the archaea in the bovine rumen, and it was concluded that more specific techniques such as quantitative PCR must be used to better determine rumen phylogeny. With the recent molecular developments such as Terminal Restriction Fragment Length Polymorphism (TRFLP) and quantitative PCR, phylogenists will in time understand the complete make up of rumen methanogens which will give better insight in how modifications in methane production can be made to ensure the best efficiency for the animal as well as the environment.

2.2.2 The Biochemistry of Methanogenesis

Methane is produced by methanogenic archaea found in numerous environments such as swamps, fresh water sediments, tundra areas, rice fields and in the gastrointestinal tracts of ruminants, and it is among the greenhouse gases that directly contribute to climate change (Deppenmeier 2002; Chaban et al. 2006). Depending on the methanogen and the environment in which it lives the pathway of methanogenesis differs. In the rumen alone there are two commonly used biochemical pathways with two different substrates: 1) CO_2 and H_2 or 2) acetate or methyl compounds. The more common pathway that converts CO_2 and H_2 to methane (Figure 2) is made up of seven reactions and employs numerous coenzymes, including methanofuran (MFR);

tetrahydromethanopterin (H_4MPT); cofactor F_{420}; cofactor F_{430}; coenzyme (CoM); and 7-mercaptoheptanoylthreonine (HS-HTP) (Rouviere and Wolfe 1988).

Rouviere and Wolfe (1988) describe methanogenesis as follows: firstly CO_2 and MFR are used to produce Formyl-Methanofuran (F-MFR) (reaction 1), the formyl group is then shifted to H_4MPT (reaction 2), next the formyl group is reduced to a methenyl group creating methenyl-H_4MPT (reaction 3), the methenyl-H_4MPT is then reduced to methylene-H_4MPT (reaction 4) and further reduced to methyl-H_4MPT (reaction 5) consuming electrons from cofactor F_{420}. The H_4MPT group is then lost by the transfer of the methyl group to CoM (reaction 6), and finally methyl-CoM is reduced to methane (reaction 7) utilizing a number of cofactors, including F_{430}, adenosine triphosphate (ATP), and flavin adenine dinucleotide (FAD) (Rouviere and Wolfe 1988). The production of methane (reaction 7) is linked to the breakdown of CO_2 (reaction 1) by the release of heterodisulfide (HTP-S-S-CoM) making the reaction somewhat cyclic. These interactions are not fully understood as of yet (Rouviere and Wolfe 1988). The overall reaction can be summarized as shown below by Deppenmeier (2002);

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O \quad (\Delta G_o' = -131 \text{ kJ/mol } CH_4)$$

The use of acetate or methyl compounds to produce methane is a less common pathway however it does contribute to overall methane emissions in the rumen. Methanogenesis using acetate as a substrate uses a shorter biochemical pathway. Acetate is first converted into carbon monoxide (CO) and methyl-CoM, the CO is then oxidized to form CO_2, with its elections being used to reduce the methyl-CoM to methane (Gottschalk 1988). Deppenmeier (2002) summarizes the reaction as follows;

$$CH_3\text{-}COO^- + H^+ \longrightarrow CO_2 + CH_4 \quad (\Delta G_o' = -36 \text{ kJ/mol } CH_4)$$

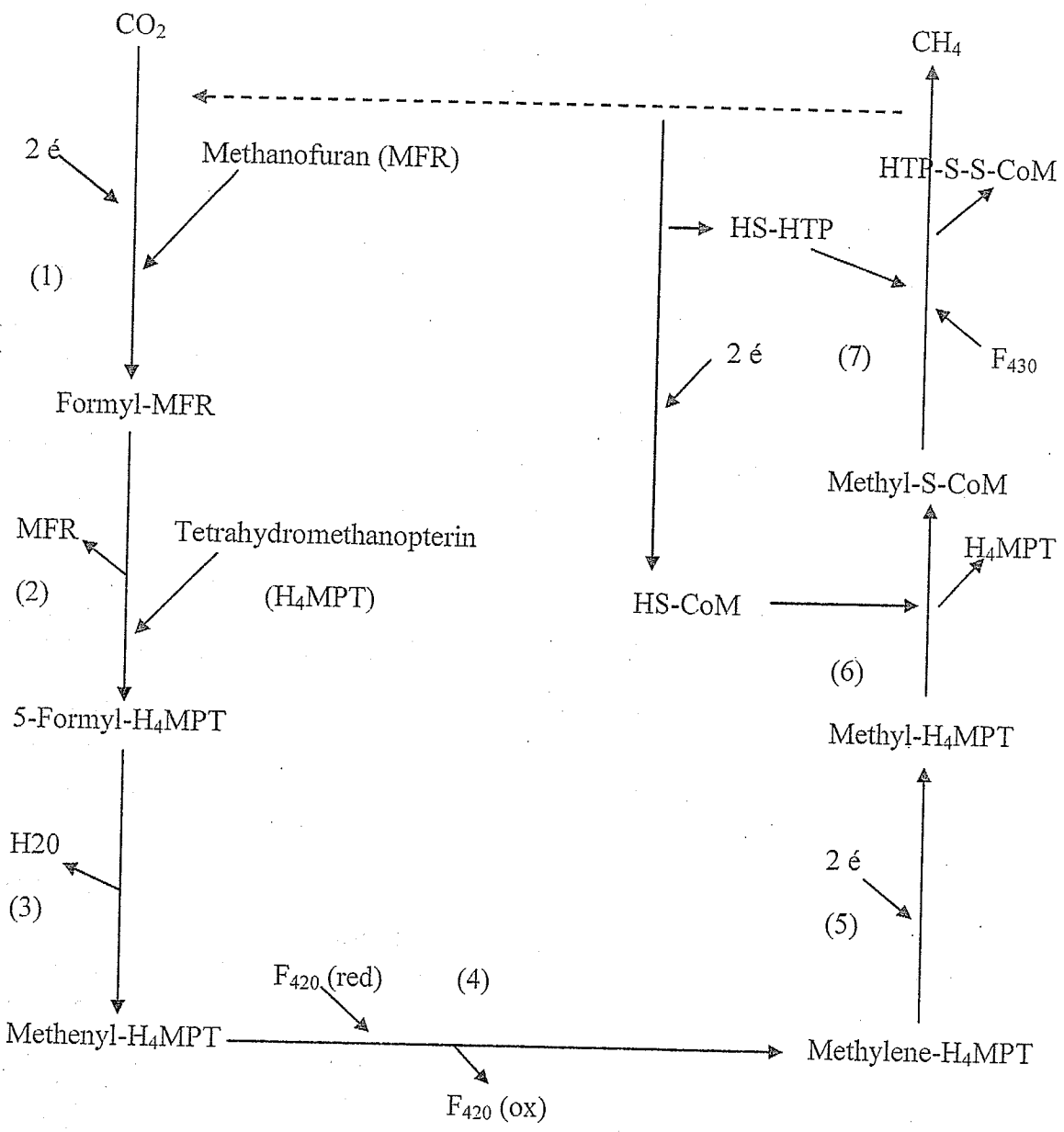

Figure 2.2: The biochemical pathway of methanogenesis

Modified from Rouviere and Wolfe 1988

Using methyl compounds for methane production is broken down into two processes. One involves the oxidation of a quarter of the methyl compounds to CO_2 which provides electrons to the other reaction which reduces the remaining methyl compounds into CH_4 (Deppenmeier 2002; Gottschalk 1988). A summary of the reactions is shown below;

$$4CH_3OH \longrightarrow 3CH_4 + 1CO_2 + 2H_2O \quad (\Delta G_o' = -106 \text{ kJ/mol } CH_4)$$

Methanogenesis represents a loss of energy and thus decreased feed efficiency for the animal. Ruminants obtain most of their energy from volatile fatty acids, and the initiative to maximize feed efficiency lies within them. The conversion of carbohydrate to either acetate or butyrate involves the production of hydrogen and furthermore the production of methane, which accounts for an ingested energy loss of typically 6% (Johnson and Johnson 1995). The pathway to convert carbohydrate to propionate is more efficient than those for either butyrate or acetate; this is because propionate has a higher enthalpy (Richardson et al. 1976) and does not result in the production wasteful methane.

Methane production is also detrimental to the environment, as ruminant methanogenesis is the largest contributor of greenhouse gas emissions in animal agriculture. Statistics show that Canadian livestock are responsible for 32% of agriculturally accountable greenhouse gas emissions, and of this 90% is from beef and dairy cattle (Kebraeb et al. 2006). For these reasons much effort has been put into finding ways to decrease the methane produced from livestock, whether it be through feed additives, such as ionophores (Odongo et al. 2007), tannins (Woodward et al. 2001), or essential oils (Beauchemin and McGinn 2006) to alter rumen microbial populations or

to utilize other mechanisms such as administering vaccines against rumen methanogens (Wright et al. 2004) to direct rumen fermentation away from methane production.

2.3 Ionophores to Reduce Methane Emissions

2.3.1 Ionophores

Ionophores are lipophilic compounds that catalyze ion movements across cell membranes. The exterior of ionophores is extremely hydrophobic, while their core is hydrophilic therefore they tend to accumulate in the lipid bilayer that is a cell membrane (Garrett and Grisham 1999). The normal movement of charged ions across a cell membrane requires high activation energy, however ionophores are capable of delocalizing the charge on ions and assist in their cross membrane movement (Russell and Strobel 1989). Thus ionophores essentially alter the movement of ions across cell membranes.

There are many different ionophores that have been approved for use, for example monensin, lasalocid, tetronasin, lysocellin, and valinomycin. These ionophores are different in their ion exchange capabilities. Some ionophores, such as valinomycin, are able to transfer one ion (most often potassium) into the cell with no exchange of H^+, these are known as uniporters (Bergman and Bates 1984). Monensin is an example of an antiporter. Antiporters have the ability to transport multiple ions at one time, as they exchange monovalent cations (sodium and/or potassium) for H^+, and the movement of these ions is based on their concentration gradients in and out of the cell (Russell and Strobel 1989). Monensin catalyzes the exchange of intracellular potassium for extracellular H^+, causing a decrease in the cellular pH. This acidification is combated by

the use of an ATPase that pumps H^+ out of the cell along with other cellular ion pumps activate to attempt to reestablish favorable ion gradients. The result is the depletion of intracellular ATP reserves from the bacterial cell which eventually causes death (Figure 3) (Russell and Strobel 1989; Callaway et al. 2003).

Ionophores are classified as antimicrobial agents as they are toxic to not only bacteria but also fungi, and protozoa and even higher organisms (Pressman 1976). These toxic effects influence rumen fermentation, as they alter the prokaryotic populations. It is the gram-positive populations that are predominately affected and results in the gram-negative populations gaining a competitive advantage (Callaway et al. 2005). Ionophore supplementation is known to inhibit populations that produce hydrogen in the rumen, and this leads to a decrease in methane production because methanogens require hydrogen to produce methane, and an increase in the propionate to acetate ratio (Russell and Strobel 1989). Yang and Russell (1993) found that monensin is inhibitory to those bacterial populations responsible for producing ammonia in the rumen; which represents a loss of dietary nitrogen. The depression of these populations in turn increases the amount of amino nitrogen and protein available to the animal (Russell and Stobel 1989; Bergen and Bates 1984). Ionophores are also known to increase feed efficiency, reduce bloat and lessen the production of lactic acid to maintain the pH balance in the rumen, preventing rumen acidosis (Tedeschi et al. 2003).

The most commonly used ionophore in ruminants today is monensin. Monensin, also known as rumensin, is produced by the fungi *Streptomyces cinnamonensis*, and was originally used as a coccidiostat in poultry, but later was also found to be beneficial in ruminants (Cheeke 2005). It was approved for use in the 1970s by the United States

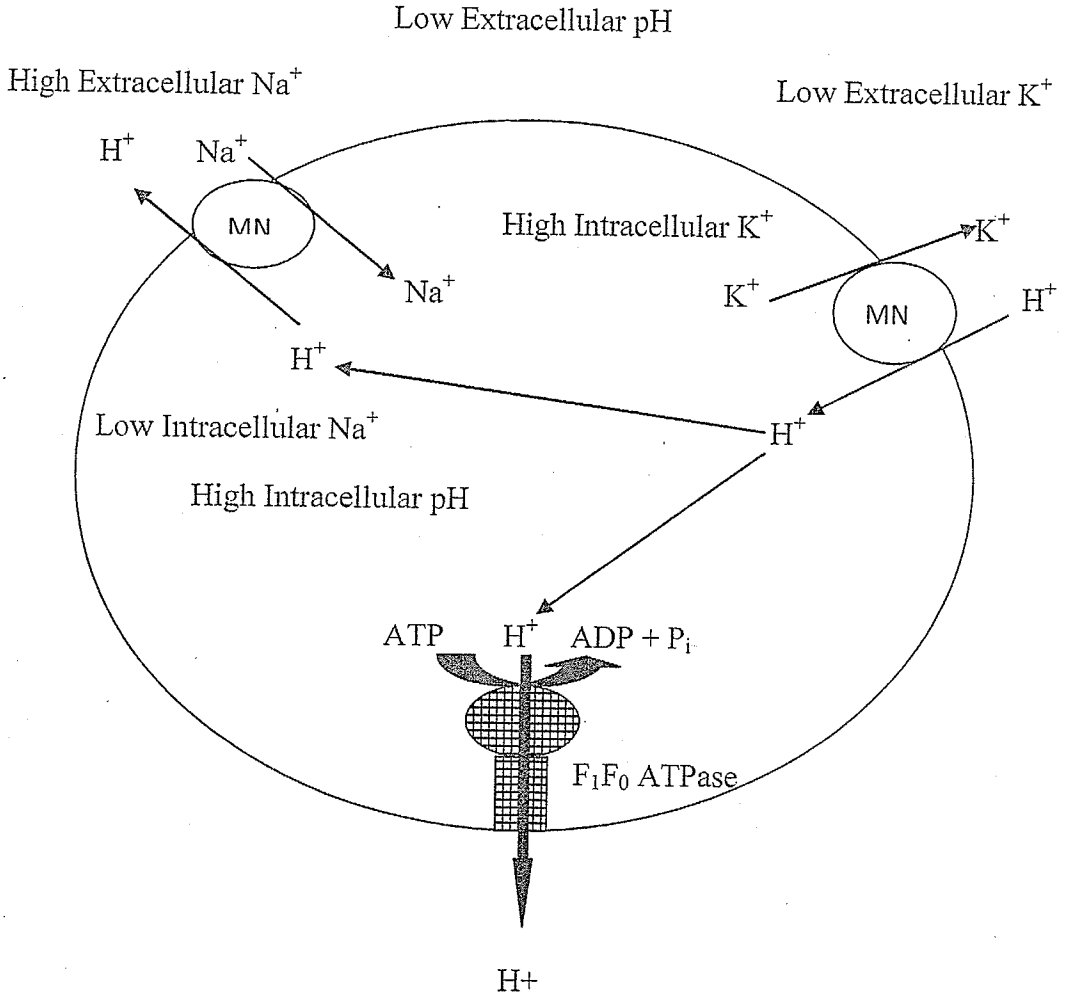

Figure 2.3: Ion shifting mechanism of monensin

Modified from Callaway et al. 2003

Food and Drug Association and since then it has been widely used in livestock production (Russell and Strobel 1989). Monensin was initially supplemented into ruminant diets as it improved feed efficiency by as much as 10% (Russell and Strobel 1989); this is attributed to changes in rumen fermentation resulting in a higher proportion of propionate to other fermentation products (Cheeke 2005). Monensin has shown to be affective in both forage and concentrate diets fed to cattle, however the ionophore reported to have a stronger response in a low forage diet (Rodrigues et al. 2004). These positive effects of ionophores on rumen fermentation has made them an integral part of livestock feed rations.

2.3.2 Ionophores and Methane Emissions

The pressure to reduce greenhouse gases has prompted a response from every industry, for animal agriculture the greatest efforts are being put forth to reduce methane emissions from ruminant livestock. Methane is a potent greenhouse gas that is known to be directly and indirectly detrimental to the environment resulting in climate change. Directly it absorbs and re-emits long-wave infrared energy back to the earth's surface and indirectly it produces CO_2, another greenhouse gas, by atmospheric oxidation reactions (Johnson and Johnson 1995; Kebreab et al. 2006). In 2002 the Canadian agriculture industry represented 8% of the total national emissions, producing 59 megatonnes of CO_2 equivalent. As of 2006 there has been a 25% increase in emissions from the Canadian agriculture sector adding 12.4 megatonnes of CO_2 equivalent since 2002 totaling 71.4 megatonnes; this is being attributed to an increase in the beef, poultry and swine industries as well as an increase of synthetic nitrogen fertilizer application on the Prairies (Environment Canada 2008). The 25% increase in the agriculture sector brings its

national total up by 0.6% to 8.6%, and until a decreasing trend is reported an even greater need for greenhouse gas solutions is present.

Ionophores have been seen to reduce methane emissions from cattle in Canada and around the world. An *in vitro* study including monensin in a submerged growing culture showed a significant decrease of methane concentration (Galindo et al. 2003). An *in vivo* study from India observed a decrease in methane emissions while feeding monensin. It was also observed that methane was indirectly proportional to feed concentrate levels and as concentrate levels went up, methane went down (Singh & Mohini 1999). A 9% decrease in methane production was measured in New Zealand when cattle were fed native pasture composed mostly of perennial ryegrass and given monensin capsules (Van Vugt et al. 2005). In Egypt, Badawy et al. (1993) showed that feeding monensin to buffalo reduced the production of methane in the rumen. Monensin was also seen to reduce methane production by 16% in steers in the United States (Thornton and Owens 1981). In Canada, Odongo et al. (2007) observed a 9% reduction (expressed as grams per kilogram of body weight) in methane emissions from dairy cattle. As well, Guan et al. (2006) reported a significant decrease in methane emissions when monensin and/or lasalocid was added to either a high forage or high concentrate diet, however this decrease was only short term.

2.3.3 Rumen Microorganisms Affected by Ionophores

Ionophores do not affect all microorganisms in the rumen. It is generally agreed that sensitivity to an ionophore is attributed to the cell wall structure of the organism (Russell and Strobel 1989). The outer membrane of gram-negative bacteria is composed of lipopolysaccharides which are endotoxins and prevent the permeation of ionophores

into the inner lipid bilayer, thus making them resistant (Russell and Houlihan 2003). Gram-positive bacteria lack this lipopolysaccharide layer in their cell membrane allowing ionophores to penetrate through the porous layer of peptidoglycan and imbed into the hydrophobic lipid bilayer leading to eventual cell death (Callaway et al. 2003). Being that gram-positive bacteria are highly susceptible to ionophores, when they are depleted the gram-negative bacteria gain the competitive advantage and fermentation shifts in their favor. However it is important to note that ionophores do affect some gram-negative bacteria, *Fibrobacter succinogenes* is initially moderately susceptible to ionophores followed by adaptaion and resistance, while *Prevotella ruminocola* though considered resistant did show changes in its physiological properties in order to grow in their presence (Newbold et al. 1993).

Studies have shown that methanogenic archaeal populations are not especially susceptible to ionophores (Van Nevel and Demeyer 1977). Van Nevel and Demeyer (1977) showed that monensin was not directly toxic to methanogens rather to those populations that break down formate to carbon dioxide and hydrogen. More recently Weimer et al. (2008) reported no change in archaeal populations when subjected to monensin feeding or monensin withdrawal. It is hypothesized that the reduction in methanogenesis by the inclusion of ionophores such as monensin is due to their harmful effect on gram-positive organisms that produce hydrogen, the required component of methane formation in ruminal fermentation, not on the methanogens themselves.

Rumen protozoa are also susceptible to ionophores (Hino and Russell 1987). Primarily *in vitro* studies showed monensin to be inhibitory to protozoal populations (Hino 1981), further research showed this to be accurate *in vivo* as well. In 1986, Dennis

et al. reported a decrease in ciliate protozoa both *in vitro* and *in vivo*; on forage or grain diets supplemented with different ionophores including monensin, however inhibition of the populations was dose dependent. An *in vivo* study by Hino and Russell (1987) showed monensin nearly eliminated all protozoa in the rumen, and Gyulai and Baran (1988) reported a decrease in protozoa in sheep while fed monensin with high forage and high concentrate diets. Protozoa populations were also seen to decrease in buffalo heifers while fed monensin (Badawy 1993). Quite recently, Martinele et al. (2008) also observed a decrease in protozoa populations in dairy cows when feeding monensin and soybean oil.

2.3.4 Ionophores Effect on Rumen Fermentation

The fermentation changes caused by ionophore implementation that have been reported most often are those of volatile fatty acid production. The major VFAs in the rumen are acetate, butyrate and propionate each having a set biochemical pathway for its production. In rumen fermentation the bacteria that produce H_2 are more apt to be those that produce the VFAs acetate and butyrate, not propionate (Russell and Houlihan 2003), and when ionophores are used in livestock diets it has been found that propionate concentrations have increased while acetate and butyrate have decreased (Singh and Mohini 1999). Callaway and Martin (1996) observed this VFA exchange when monensin was supplemented into the diet, the ratio of acetate to propionate decreased. Similar findings were reported by Singh and Mohini in 1999, showing an increase in propionate levels and a decrease in not only acetate but butyrate as well. In another study when monensin was used to measure its effect on ciliate protozoa, protozoa numbers were correlated with the proportion of propionate in rumen fluid and it was suggested that

the defaunation of the ciliates is related to the reduction in methane and the shift in VFAs towards propionate (Martinele et al. 2008).

The alteration in VFA formation by monensin is the explanation for the increase in feed efficiency for animals. Monensin shifts the production of VFAs towards propionate and away from acetate, which results in less energy being lost for the production of unnecessary byproducts such as methane (Moss et al. 2000). With the state of the world's food supply, agriculture is under pressure to produce high levels of outputs such as meat and milk, and using monensin in feed rations to increase efficiency has helped to meet these demands.

2.3.5 Effects of Ionophores and pH in the Rumen

The alteration in rumen microbial populations and fermentation that occur with ionophores may be coupled to diet and rumen pH. Rumen microorganisms have specific parameters that allow for optimal growth, and depending on the carbohydrate (forages or concentrate) being fed, the rumen environment changes significantly (Nagaraja and Titgemeyer 2007; Hungate et al. 1952). Diets high in concentrate carbohydrate, such as barley provides large amounts of energy to the animal but they must be properly rationed to avoid severe acidosis in the rumen. The results of feeding a high grain diet are a shift in rumen microflora towards fast growing, amylotic, lactic acid producing bacteria, such as *Streptococcus bovis and Lactobacillus spp.* and a depletion of cellulolytic bacteria (Cheeke 2005) and protozoa (Nagaraja and Titgemeyer 2007). Lactic acid is approximately 10 times more acidic than volatile fatty acid and its accumulation in the rumen results in a decrease in pH ranging from 0.4 pH points to the suboptimal level for rumen function of less than 5.6, and overtime to as much as 1.5 pH points below pH 5.0,

at this point the animal suffers acute acidosis and may not survive (Nagaraja and Titgemeyer 2007). It is important to note that reduction of pH by 1.0 log unit is equivalent to a 10-fold increase in H^+ concentration in the rumen, thus what seems like a small change is in fact very significant (Nagaraja and Titgemeyer 2007).

Some organisms are able to adjust to the acidification of the rumen. Research indicates that the acid tolerant organisms allow their intracellular pH to decrease as a function of the extracellular pH, *S. bovis*, *S. ruminatium*, and *P. ruminicola* all demonstrated this phenomenon when grown in an environment with a decreasing pH (Russell 1991). As well *Lactobacillus spp.* showed a decrease in intracellular pH as a response to extracellular pH and was able to grow in acidic conditions (Nannen and Hutkins 1990).

The more acidic rumen environment that comes as a result of concentrate feeding independently affects rumen flora but may also contributes to the effect ionophores have in the rumen. Chow and Russell (1990) showed that monensin and lasalocid are more effective when the pH is low in the rumen, because the carboxyl group on the ionophore is near the surface and its state of ionization is dependent on pH. If the pH is lower than the pK_a of the ionophore it does not become ionized and it can dissipate ion gradients and penetrate the cell membrane with ease (pK_a of monensin is 7.95) (Russell and Houlihan 2003).

Ionophores can help to combat the acidification of the rumen on high grain diets. Monensin and lasalocid have been shown to reduce acidosis in the rumen as they are effective against the two largest lactic acid producers, *Lactobacillus spp.* and *S. bovis*, but are not effective to the lactate fermenting organisms in the rumen (*Megasphaera*) (Dennis

and Nagaraja 1981). Therefore ionophore supplementation can be considered a preventative strategy in acidosis, working to maintain healthy rumen function

2.4 Ionophore Adaptation & Resistance

2.4.1 Antibiotic Supplementation in Feeds

Antimicrobial resistance has become a major issue in human and animal health with the development of multi-drug resistant bacteria. The use of antibiotics in animal production at sub-therapeutic levels has been a common practice for more than 50 years; as it acts as a growth promotant for young animals and has prevented and/or alleviated disease (Jukes 1972). The concern with using antibiotics in animal feeds was the possibility of generating resistant organisms and therefore making the treatment of disease, for humans or animals, much more difficult (Cheeke 2005). Upon generating resistance to an antibiotic it is quite simplistic for the resistance gene to be transferred to other organisms, as the gene is normally found on a plasmid. A plasmid is a piece of genetic material that can exist and replicate independent of a chromosome (Prescott et al. 2002) and the fear is the resistance gene will eventually be subjected to humans through feeding. Another concern is the possibility of antibiotics remaining in animal products which are ingested by humans; however this concern has lessened with the implementation of required antibiotic withdrawal times, a designated period of time after antibiotic administration for the animal to be sent to market. As of January 1, 2006, the European Union has banned the supplementation of all antimicrobials, including monensin, into animal feeds and restricting their use for disease treatment only, this is in

the hopes of preventing the development of drug resistant bacteria and other organisms in livestock and in humans (Europa 2005).

Ionophores are among the feed additives that have antimicrobial capabilities and the possibility of generating resistant organisms. The best explanation for ionophore resistance and sensitivity in bacteria is dependent on cell wall structure, as previously explained; however some initially susceptible rumen bacteria have been seen to develop resistance or adapt to the ionophore treatment. Chen and Wolin (1979) showed some *Bacteroidetes* strains to be initially stunted by monensin or lasalocid treatment but eventually became ionophore resistant. Bacterial species that are normally susceptible to ionophores but grow very rapidly such as *Streptococcus bovis* can actually outgrow the presence of monensin, and a higher concentration of the ionophore must be added to have any effect on the populations (Callaway et al. 1999).

2.4.2 Mechanism of Adaptation

Studies on ionophore adaptation in the rumen have shown varied results. The mechanism of adaptation is currently being heavily studied and has presented many theories how it occurs. It is known that susceptibility to ionophores is related to the movement of ions; therefore a thought mode of adaptation would be an increase in ion pump activity by the cell (Russell and Strobel 1989). Another possible route for ionophore adaptation is the use of membrane bound translocases which acts to completely remove ionophores from the bilayer membrane; this would certainly be a viable mechanism to achieve resistance and would account for the initial susceptibility followed by a sharp increase of some rumen bacterial populations (Lewis et al. 1994; Callaway et al. 2003); however it is yet to be verified that an ionophore translocase is

present in ruminal bacteria. A study by Callaway et al. (1999) showed that highly sensitive low G + C (guanine + cytosine) gram-positive bacteria can significantly adapt to monensin upon repeated exposures to the ionophore, these bacteria included *Streptococcus bovis*, *Clostridium aminophilum*, and *Selenomonas ruminantium*.

Adaptation was also observed in an *in vitro* and *in vivo* study by Newbold et al. (1993) showing gram-negative species being initially susceptible to monensin but quickly developing resistance. Newbold et al. (1993) also reported the development of ionophore cross resistance within the gram-negative bacteria, therefore when resistance was developed to one ionophore the bacteria showed resistance to others it was subjected to. The hypothesized mode of action for this resistance was that *F. succinogenes* as well as *P. ruminicola* prevented ionophores from entering the cell envelope by shrinking the size of its porins and non-selectively eliminating the entry of large molecules (Newbold et al. 1993). Many thoughts are present on how exactly adaptation to ionophores occurs in the rumen, but at this time no firm conclusions can be drawn. The question is whether or not this ionophore resistance will eventually lead to resistance to other antibiotics in animals and/or humans.

A study was performed to measure the resistance gained by bacterial species subjected to monensin by determining potassium depletion in their respective cells. Lana and Russell (1996) used potassium as a measure of sensitivity because ionophore induced potassium depletion was concentration dependent and it was possible to explain the relationship with saturation constants. They found that ionophores have a direct effect on bacterial populations *in vivo*, but there is a very large population of bacteria that are naturally resistant to ionophore treatment and are able to rapidly replace the ionophore

sensitive bacteria as a greater living space is created. Bacteria in animals fed ionophores showed a greater level of resistance compared to the control animals (which did show some resistance), however even those bacteria that became resistant still lost intracellular potassium when given high levels of ionophore *in vitro* (Lana and Russell 1996). These finding support the theory that adaptation to ionophores in rumen bacteria is a fundamental feature of physiology and not a transferable genetic element.

2.4.3 Ionophores and Antibiotics

The concern with using ionophores in animal feeds is not only the development of bacterial resistance but resistance to whole classes of antibiotics. These concerns are very viable in that multi-drug resistant bacteria have developed and have made treatment programs more difficult in animal and human health. But do ionophores lead to resistance to other antibiotics? Research done by Edrington et al. (2003) showed that they may not. In fact it was found that the number of isolates of *Escherichia coli* O157:H7 resistant to streptomycin in sheep were less in those treated with bambermycin as compared to control animals. The sheep fed ionophores (monensin, laidlomycin propionate or bambermycin) also showed no effect on pathogenic *E. coli* or *Salmonella* in fecal sheddings or in gastrointestinal juices (Edrington et al. 2003). And it was concluded that ionophore supplementation had no effect on the antimicrobial susceptibility of *E.coli* or *Salmonella enteritidis* (Edrington et al. 2003). Being that ionophores do not play an active role in adaptation of pathogenic organisms to antibiotics, they may still be used in livestock diets as a means of increasing production efficiency and as well be used as a strategy to reduce methane production in cattle.

2.5 Summary

The rumen is a highly complex and ever changing ecosystem, one that has attracted much scientific interest. Cattle are known to produce methane as a byproduct of fermentation in the rumen, and this contributes to the harmful effects of climate change. A solution to this problem if only short term has been the implementation of ionophores such as monensin in to the diets of cattle. Monensin has been shown to decrease methane emissions from cattle (Guan et al. 2006), this may greatly impact the agriculture industry as methane from livestock contributes largely to its total emissions. As well with the ever growing human population production demands have forced the agriculture industry to use new methods to increase the amount of food they produce. Feed efficiency does improve when ionophores are supplemented in the diet which not only increases production but lowers cost. Monensin has been useful in attaining these environmental and production goals while maintaining strong and healthy animals.

The use of ionophores does affect rumen microbiology and fermentation pathways, by depleting certain microorganisms, typically gram-positive bacteria and protozoa, allowing others such as the gram-negative bacteria to thrive. Some organisms have adapted to the ionophore treatment, however there does not seem to be development of antimicrobial resistance, and therefore should not be feared for use in the livestock industry.

3.0 HYPOTHESES AND OBJECTIVES

The objective of this study was to evaluate the microbial population adaptation in the rumen when treated with the ionophore monensin. Monensin is known to have antimicrobial capabilities predominately towards gram-positive bacteria, and therefore change the prevalence and abundance of certain microbial species in rumen, thus modifiying rumen function. Previous research by Guan et al. (2006) found monensin did have an inhibitory effect on methane production from steers; however it was short term. Methane emissions returned to baseline levels after 4 to 6 wks of ionophore supplementation. The reason for this adaptation likely lies in the dynamic nature of the rumen ecosystem and microbial adaptations to monensin is the focus of this research. Thesis objectives were:

1. To characterize the microbial adaptations in the rumen when cattle are fed monensin.
2. To characterize the structure of the rumen microbial community when it adapts to monensin.
3. To quantify species of microorganisms that may be key to methanogenesis and describe their shifts in relation to methane emissions during adaptation.

The hypotheses tested in this research were:

1. The action of monensin is not directly on methanogens but on hydrogen producing species of bacteria.
2. Because monensin acts on gram-positive bacteria we hypothesize that it is these bacteria that adapt to monensin.

3. We further hypothesize that the adaptation will occur with the hydrogen producing gram-positive bacteria.
4. We hypothesize that monensin does not act directly on methanogens, and consequently there should be no change in the structure of this population.

4.0 MANUSCRIPT

Rumen microbial community analysis and methanogenesis during adaptation to monensin

4.1 ABSTRACT

The aim of this study was to characterize the adaptation of rumen microbial populations and methane emissions to ionophores when fed to cattle over a 12 wk period. Methane emissions from ruminants contribute approximately 90% of the greenhouse gas emissions from animal agriculture (Kebreab et al. 2006). The gram-positive specific ionophore antibiotic, monensin, may lower methane production in the rumen, however the effects appear to be short term (weeks) and there is a rebound effect in which emissions return to baseline levels. We hypothesized that gram-positive bacteria, but not methanogenic archaea, adapt to monensin and that the methane emission rebound can largely be explained by adaptation of gram-positive hydrogen producing bacteria. Rumen samples were taken weekly for 12 wk from cattle fed either a high grain or a high forage diet with or without monensin (Guan et al. 2006). Methane emissions were simultaneously measured. Microbial populations were characterized using terminal restriction fragment length polymorphisms (TRFLP) analysis. Key microbial species were quantified with real-time PCR (RT-PCR). TRFLP indicated that at the phylum level community structure was relatively stable showing no statistical difference ($P < 0.05$) between treatment or diet as well as no difference over time. There was a noticeable prevalence of members of the phylum Verrucomicrobia. No members of this phylum have been cultured from the rumen but may be involved in polysaccharide degradation or anaerobic methane oxidation. RT-PCR indicated that adaptation occurred partly within the hydrogen producing gram-positive bacteria on the concentrate diet and the effects on methanogens were strongly correlated with these population shifts. In the

forage diet the adaptation to ionophores also occurred but modulation of gram-positive bacteria appeared to be much more subtle. The methanogenic archaea were not directly affected by monensin, the observed abundance changes were related to the decrease in the abundance of ciliate protozoa and the available hydrogen in the rumen. Methane emissions returned to baseline levels two wk earlier in grain than forage diets and this is likely the result of higher turnover rates in grain based rations. A low correlation between methane levels and temperature showed that the influence of temperature on the data was not large but could not exclude it.

4.2 INTRODUCTION

Ruminants contribute approximately 90% of the greenhouse gas emissions from animal agriculture (Kebraeb et al. 2006). Lower rates of production are obtained in animals fed primarily on forages as compared to those fed high grain diets. However, animals on high forage diets take much longer to reach market weights and focus has been placed in reducing greenhouse gas emissions in finishing cattle fed high grain diets (Lana et al. 1997; Weimer et al. 2008). Various dietary manipulations have been investigated including ionophores (Odongo et al. 2007), essential oils (Beauchemin and McGinn 2006), tannins (Woodward et al. 2001), and even vaccines (Wright et al. 2004).

One of the most promising approaches is the use of the ionophore antibiotic monensin. Initial studies demonstrated that a substantial reduction in methane emissions could be achieved, but these studies only measured methane for relatively short (one month) periods (McGinn et al. 2004). However, feedlot cattle are usually fed for two to

three months so emissions over a longer period are more relevant. Guan et al. (2006) demonstrated that ionophores initially reduced methane emissions in feedlot cattle fed either high grain or high forage diets. This reduction was only evident for four weeks on the concentrate diet and six weeks on the forage diet, after which the methane levels again returned to baseline levels.

Clearly, the rumen microbial ecosystem adapted to the inclusion of ionophores in the diet. Callaway et al. (1999) reviewed the literature on monensin supplementation, and concluded that some members of the phylum Firmicutes adapted to monensin. These rumen bacterial species are gram-positive and it is well documented that *in vitro* the mode of action of monensin is towards the gram-positive cell wall (Callaway et al. 2003). Newbold et al. (1993) demonstrated that monesin might also cause resistance in the rumen gram-negative bacterium *Prevotella ruminicola*, a member of the phylum Bacteroidetes. The mechanism of adaptation was hypothesized to be the shrinking of porins in the cell membrane.

This research evaluated the adaptation that occurs in the rumen microbial community to monensin over a 12 week period. The samples collected from the study by Guan et al. (2006) were used and microbial community analysis was conducted using terminal restriction fragment length polymorphisms (TRFLP). Specific microbial populations including archaea and protozoa were evaluated using real-time PCR.

4.3 MATERIALS AND METHODS

4.3.1 Sample Collection

A previous experiment performed at the University of Manitoba divided 24 Angus steers into four dietary treatments. Treatments were high forage or high concentrate, with or without monensin, and methane emissions were measured using the SF_6 technique (Boadi et al. 2002) over a 16 wk period (Guan et al. 2006). The time points selected for microbial analysis were chosen to reflect the time-course of methane decline and adaption over a critical 12 wk period as observed by Guan et al. in 2006 (Fig. 4.1A & 4.1B). Frozen rumen fluid samples from three randomly selected animals for each of the four diets at six specific time periods were selected for study (Table 4.1).

4.3.2 DNA extraction, PCR Amplification and Restriction Digest

Frozen rumen fluid samples were thawed at 32 °C for 15 min. and were then centrifuged at 10,000 x g for 20 min. The supernatents were discarded and the pellet was re-suspended in 0.9% saline, and frozen at -20 °C. DNA was extracted for downstream processing using the ZymoResearch Fecal DNA Extraction Kit (Orange, CA), following the manufacturers protocol. The bacterial 16S rRNA gene was then amplified using PCR with the specific primers 27f (5' - AGAGTTTGATCCTGGCTCAG - 3') and 1100r (5' - TTGCGCTCGTTGCGGGACT - 3') (Lane 1991). The reaction mixture was made up of 16.9 μL autoclaved distilled water, 2.5 μL 10X reaction buffer (Lucigen, Middleton, WI), 2 μL 25mM $MgCl_2$, 0.5 μL 10mM PCR nucleotide mix (Fisher Scientific, Ottawa, ON), 0.5 μL of the forward and reverse primers and 0.1 μL of Taq DNA polymerase (Lucigen, Middleton, WI). The PCR program consisted of a 5 minute initial denaturation step at

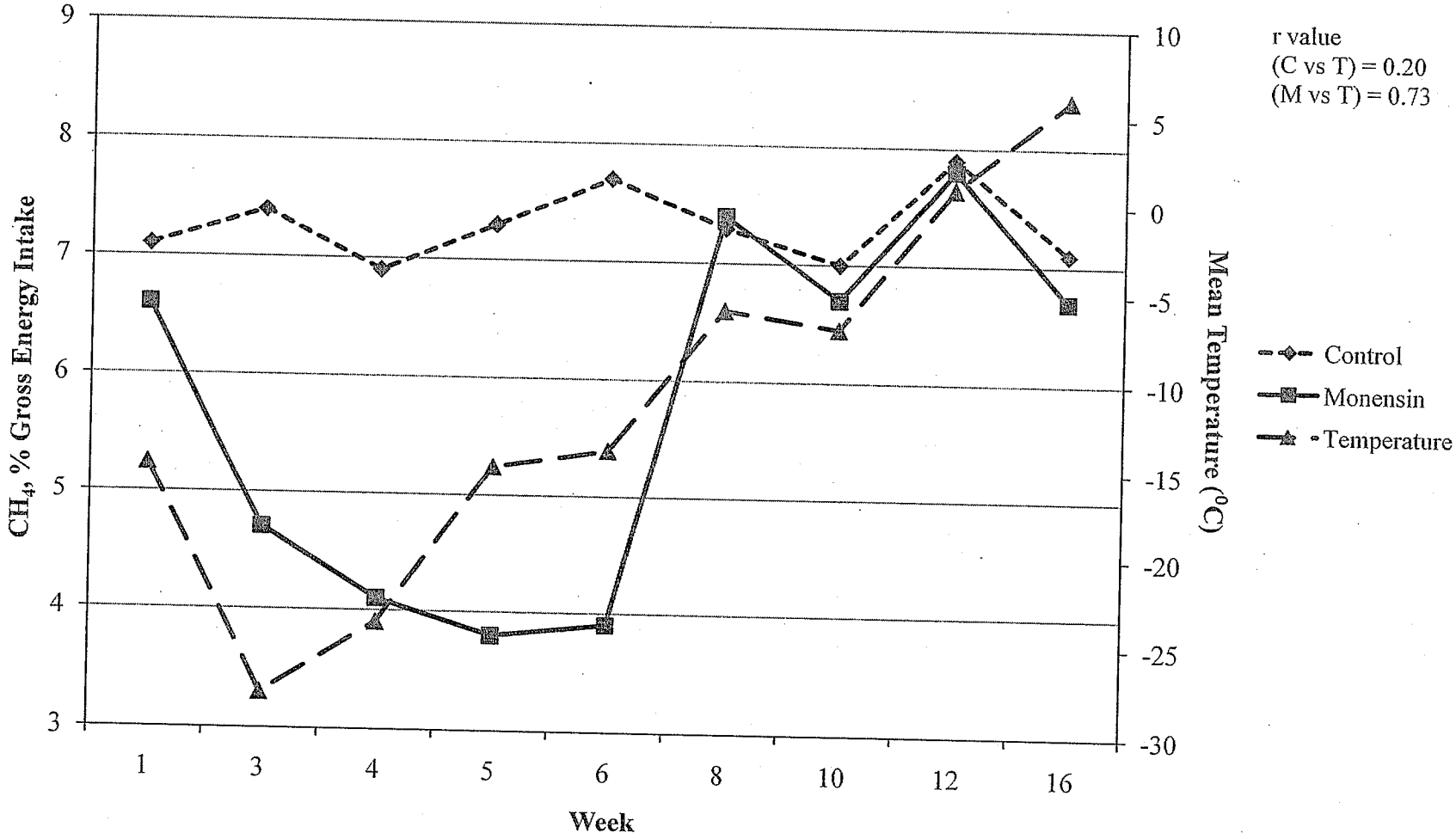

Figure 4.1A. Methane emissions as a percent of gross energy intake from animals fed a high forage diet correlated with mean temperatures over time. (r – correlation; coefficient; C – control; M – monensin; T – temperature)

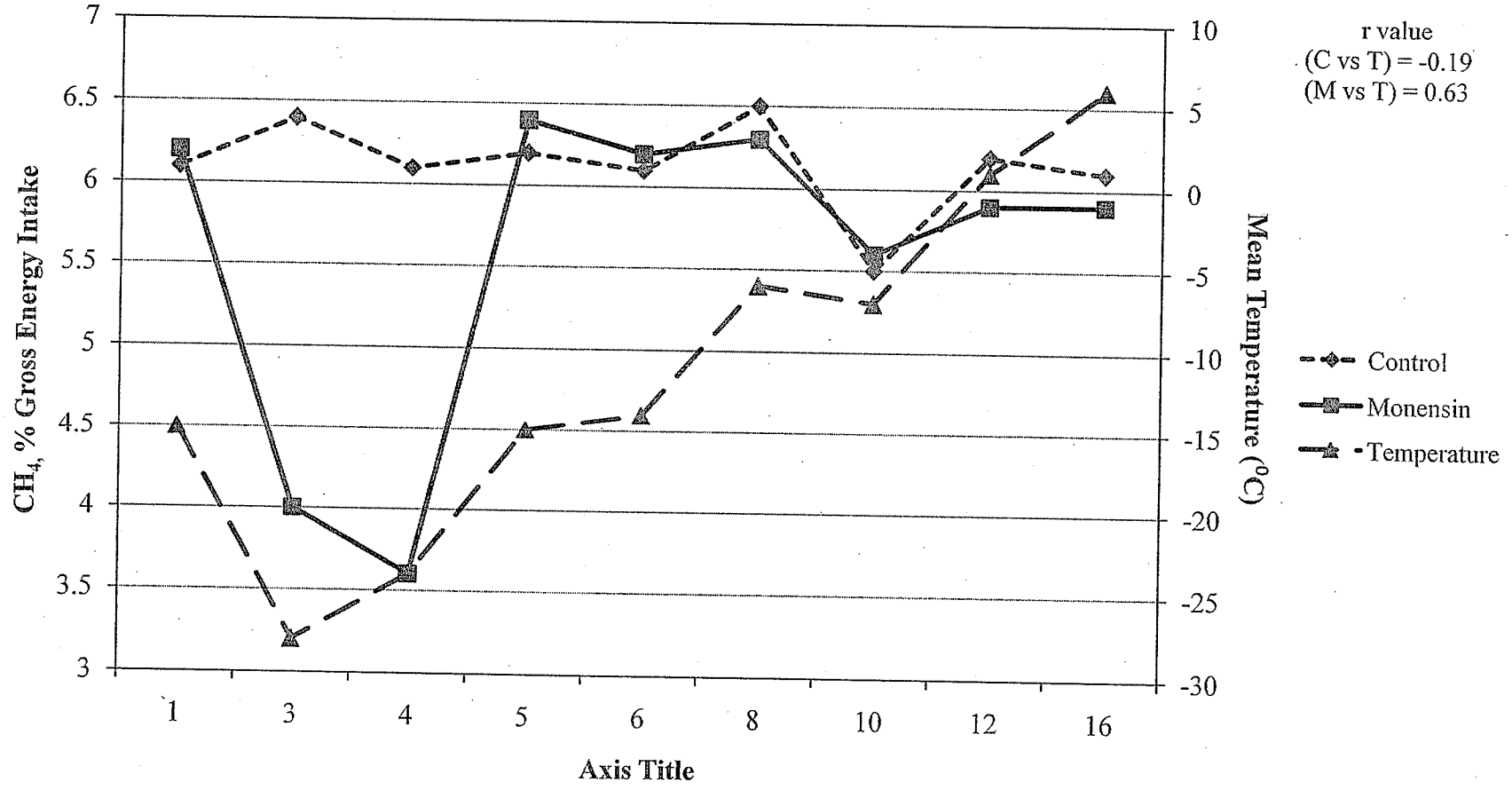

Figure 4.1B. Methane emissions as a percent of gross energy intake from animals fed a high concentrate diet correlated with mean temperatures over time. (r – correlation; coefficient; C – control; M – monensin; T – temperature)

Table 4.1: Rumen fluid samples randomly chosen for study

Diet	Animal Number
High Forage Control	4, 9W, 40
High Forage Monensin	25, 56, 84
High Concentrate Control	16, 19, 46
High Concentrate Monensin	12, 22, 55

Samples taken from Guan et al., 2006

94 °C, 36 cycles of 1 minute denaturation at 94 °C, 1 minute for primer annealing at 61 °C, and 2 minutes for extension at 72 °C, a 5 minute final extension period at 72 °C and the final product was held at 4 °C. The archaeal 16S rRNA gene was amplified with the specific primers 109f (5' - ACKGCTCAGTAACACGT - 3') and 934r (5' - GTGCTCCCCCGCCAATTCCT - 3') (Microbial Community Analysis III 2007) using the following program; initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 1 minute, annealing at 51.9 °C for 30 seconds and extension at 72 °C for 2 minutes, and a final extension at 72 °C for 10 minutes; the product was held at 4 °C. The reaction mixture for the archaeal PCR was the same for that of the bacterial reaction. For both the bacterial and archaeal amplifications the forward primers (27f and 109f) were fluorescently labeled with a cyanide based dye (WellRED oligo, Sigma-Aldrich, Oakville, ON) so they could be detected by capillary electrophoresis using the Beckmann Coulter CEQ 8800 (Beckman Coulter Inc., Fullerton, CA).

The PCR products were then digested to obtain terminal restriction fragments (TRFs). This was performed using the restriction enzymes *Hha*I (GCG^C) (Promega, Madison, WI) and *Mse*I (T^TAA) (New England Bio Labs, Ipswich, MA) for the bacterial and archaeal genes respectively. A mixture of 1 μL restriction enzyme, 0.2 μL 10 mg/mL bovine serum albumin, and 2 μL 10x reaction buffer was made for each sample and incubated at 37 °C overnight to maximize the reaction. The restriction enzymes were chosen using the Enzyme Resolving Power tool in MICA III (2007), and were those resulting in the maximum number of fragments.

The restriction products (approximately 20 μL) were then desalted using a mixture of 0.25 μL 2 mg/mL glycogen and 2.1 μL 3M NaOAc pH 5.2. To the mixture

57 µL of 95% v/v ethanol was added and centrifuged at 15, 000 x g for 15 min at 4 °C. The supernatant was discarded and the pellets were then rinsed twice with 100 µL 70% v/v ethanol, after each rinse the samples were centrifuged at 15, 000 x g for 5 min at 4 °C, the supernatant was discarded and then left to air dry. Once dry, the samples were re-suspended in sample loading solution (Beckmann Coulter Inc., Fullerton, CA) for further downstream analysis.

4.3.3 Terminal Restriction Fragment Length Polymorphism (TRFLP)

A master mix of 29.25 µL sample loading solution and 0.75 µL of 1000 basepair (bp) DNA size standard (Beckman Coulter Inc., Fullerton, CA) was made, 30 µL of this master mix was pipetted into the sample tray for the CEQ 8800. To the master mix 3 µL of the PCR product was added followed by one drop of mineral oil to prevent the samples from evaporating; all samples were run in triplicate. The machine required the use of a wetting tray, filled with approximately 10 mL of double distilled Milli-Q water, a tray for separation buffer filled for each sample and approximately 5 mL of gel for the capillaries to perform properly. The CEQ software (Version 9.0; Beckman Coulter Inc., Fullerton, CA) was used to analyze the data, when analyzing a minimum level of fluorescence was defined in the machine to eliminate any unwanted background noise. The background was determined using a negative control PCR product. The fragment data output came in two forms, an electropherogram; a series of colored peaks representing the microbial community, and a numerical table which includes the size (base pairs) and height of peak. The height of each peak represents a measure of the proportion of each population in the community relative to each other. Samples which did not run properly based on these outputs were deleted and repeats were performed.

4.3.4 Bioinformatic Analysis of Bacterial TRFLP Data

The CEQ software cannot provide taxonomic information to the family level that was sought, and therefore a highly specific bioinformatic analytical pipeline utilizing numerous internet based tools was used as previously described by Seperhi et al. (2007) and Bhandari et al. (2008). Firstly the Ribosomal Database Project (RDP, Cole et al., 2008; Accessed August 2008) was employed because it is a source for thousands of sequences known to reside in the rumen, using these sequences a highly specific bacterial rumen database to study microbial ecology was developed (Seperhi et al. 2007). The database was uploaded into Microbial Community Analysis III program (MICA III) and cross referenced with the bacterial primers and restriction enzyme used. The output is a reference database specific to our primers and restriction enzyme. This reference database and the bacterial TRF data were then uploaded in to the Phylogenetic Assignment Tool (PAT; Kent et al. 2003) and cross referenced as described by Seperhi et al. (2007). The output from PAT provided GenBank accession numbers to identify which organisms represented each TRF. The GenBank accession numbers were input into the RDP hierarchy browser, which presented the phylum, class, order and family of each match as well as their prevalence in the rumen.

4.3.5 Primer Selection and Real Time Polymerase Chain Reaction

The primers used for Real Time Polymerase Chain Reaction (RT-PCR) are listed in Table 4.2 (Khafipour et al. 2008). The primers for the specific organisms were chosen based on the community profile given by the TRF data. The primer oligonucleotides were selected from supporting literature (Khafipour et al. 2008) and were synthesized by University Core DNA Services (University of Calgary, Calgary, AB).

The three DNA samples extracted from rumen fluid for each diet were pooled for each time period, giving 4 diet samples over 6 weeks and 24 total samples to run for each primer. The concentration of the pooled samples was measured at a wavelength of 260nm using the Beckman Coulter DU 800 spectrophotometer (Beckmann Coulter, Fullerton, CA) by adding 10 μL of sample into 90 μL distilled water. The spectrophotometer was blanked using 100 μL of distilled water. The samples were subsequently diluted 5 times to attain the working concentration of approximately 5 ng/μL. A standard curve was also established to run with each primer set. DNA from one animal for each diet for every week was pooled and the concentration was measured using the spectrophotometer as explained above. The sample concentrations were measured at 37.3 ng/ μL and diluted 5 times to a concentration of 7.46 ng/ μL, than seven two-fold series dilutions were performed to a concentration of 0.0582812 ng/ μL. These samples were run with each primer as a tool to compare the experimental samples against.

RT-PCR was performed using the AB 7300 system (Applied Biosystems, Foster City, CA) and the sequence detection software (Version 1.3; Applied Biosystems, Foster City, CA). Each sample was run in triplicate at a reaction volume of 25 μL in a 96-well optical plate covered by an adhesive film to prevent drying (Applied Biosystems, Foster City, CA). The reaction mixture was as follows; 5 μL Water, 12.5 μL Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1.25 μL of the forward primer and 1.25 μL of the reverse primer, all multiplied by the total number of samples set to run. Master mix was vortexed briefly and 20 μL was pipetted into each well of the 96-well plate, to this 5 μL of pooled DNA sample was added and ran in the thermocycler. The program for each of the bacterial primers were different than the methanogenic archaea and ciliate protozoa, with the

Table 4.2. Primers used for Real Time Polymerase Chain Reaction

Target Organism	Primer Set	Primer Sequences (5' → 3')	Tm (°C)	G+C %	Amplicon Size (bp)	Source of Primer
Eubacteria	341-357F	CCTACGGGAGGCAGCAG	55.2	70.6	189	Muyzer et al. 1993
	518-534R	ATTACCGCGGCTGCTGG	56.2	64.7		
Ciliate Protozoa	UPorCil 1F	GCTTTCGWTGGTAGTGTATT	50.2	20.0	234	Sylvester et al. 2004
	UPorCil 1R	CTTGCCCTCYAATCGTWCT	50.4	47.4		
Methanogenic Archaea	MB1174f	GAGGAAGGAGTGGACGACGGTA	60.6	59.1	232	Ohene-Adjei et al. 2007
	Arch1406-1389r	ACGGGCGGTGTGTGCAAG	60.0	66.7		
Streptococcus bovis	SBovis1F	TTCCTAGAGATAGGAAGTTTCTTCGG	57.9	42.3	127	Steveson and Weimer 2007
	SBovis1R	ATGATGGCAACTAACAATAGGGGT	57.9	41.7		
Lactobacillus spp.[1]	Ulac16S1F	AGCAGTAGGGAATCTTCCA	51.5	47.4	345	Walter et al. 2001
	Ulac16S1R	ATTCCACCGCTACACATG	51.1	50.0		Lan et al. 2004
Ruminococcus flavefaciens	RumFla1F	CGAACGGAGATAATTTGAGTTTACTTAGG	57.5	34.5	132	Denman and McSweeney 2006
	Rum Fla1R	CGGTCTCTGTATGTTATGAGGTATTACC	59.3	42.9		
Ruminococcus albus	RumAlb1F	CCCTAAAAGCAGTCTTAGTTCG	54.3	45.5	176	Wang et al. 1997
	RumAlb1R	CCTCCTTGCGGTTAGAACA	53.8	52.6		
Selenomonas ruminantium	SelRum1F	GGCGGGAAGGCAAGTCAGTC	60.4	65.0	83	Khafipoor et al. 2008
	SelRum1R	CCTCTCCTGCACTCAAGAAAGACAG	61.1	52.0		
Prevotella ruminocola	PreRum92862F	GCGAAAGTCGGATTAATGCTCTATG	58.5	58.5	78	Khafipoor et al. 2008
	PreRum92862R	CCCATCCTATAGCGGTAAACCTTTG	59.3	59.3		
Butyrivibrio fibrosolvens	ButFib2F	ACCGCATAAGCGCACGGA	58.8	61.1	65	Stevenson and Weimer 2007
	ButFib2R	CGGGTCCATCTTGTACCGATAAAT	55.7	45.8		

[1] Primer Sets were made to match 14 *Lactobacillus spp.* Including *Lactobacillus acidophilus* (1), *Lactobacillus crispatus* (1), *Lactobacillus delbrueckii* (1), *Lactobacillus fermentum* (1), *Lactobacillus helveticus* (2), *Lactobacillus nodensis* (1), *Lactobacillus paralimentarius* (1), *Lactobacillus pontis* (3), *Lactobacillus sp.* (3), and 154 unclassified bacteria

annealing temperature changing and adding a longer extension; for the bacterial primers the program was an initial denaturation of 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, and annealing at 60 °C for 1 minute. The methanogenic archaea and the ciliate protozoa followed the same denaturation protocol but an annealing/extension step of 63 °C for 30 seconds/72 °C for 30 seconds and 54°C for 30 sec/72°C for 1 minute was applied respectively.

The amplification efficiency (E) was determined using the slope of the standard curve. The standard curve was created by plotting the threshold cycle (CT) versus logarithmic values of different pooled DNA concentrations using the following equation (Denman and McSweeney, 2006):

$$E=10^{-1/slope}$$

Relative quantification was done using the Relative Standard Curve Method as documented in the Applied Biosystems User Bulletin #2, 1997 (Applied Biosystems 2001), by comparing the experimental pooled samples to the standard curve.

4.3.6 Clustan Analysis

The archaeal binary TRF data was analyzed using the Clustan Graphics 7 (Edinburgh, Scotland) cluster analysis software. Clustan produces hierarchical clusters of the data grouping samples together based on similarity. The TRF data compared each diet, pooled data from each individual animal, against one another across each time point using the Jaccard's similarity coefficient clustering method, which does not consider the absence of a characteristic between two samples as a similarity. Multiplying the Jaccard's similarity coefficient by 100 gives the percentage of similarity between the two tested

samples. The Clustan software provided a quick and easy method of looking at the relationships of the archaeal populations in the rumen over diet and time.

4.3.7 Data Analysis

The Fisher Exact Test ($P < 0.05$) was used to test the significance between diet and monensin treatment for the TRFLP prevalence data. A statistical t-test ($P < 0.05$) was performed on RT-PCR data (Forage period 2: $n = 9$; period 3: $n = 6$; Concentrate period 2: $n = 6$; period 3: $n = 9$) to test significance between monensin and control samples in one time period as well as significance between time periods for each microorganism.

4.4 RESULTS

4.4.1 Methane Emissions and Temperature

The correlation graphs of the methane levels as a percent of gross energy intake and mean temperature (°C) for the forage (Fig 4.1A) and concentrate (Fig 4.1B) diets showed that temperature and methane levels did show a positive correlation. However the correlation was low (0.19 – 0.73) and therefore it cannot be said that temperature alone is responsible for the effects on methane emission.

4.4.2 Terminal Restriction Fragment Length Polymorphism

TRFLP analysis of rumen samples indicated that the major bacterial phyla for both the forage and concentrate diets (Table 4.3) were the Firmicutes (>80%), Proteobacteria (~5%), Bacteroidetes (~0.5%), Actinobacteria(~2.5%), and Verrucomicrobia (~3.5%). The unclassified bacteria made approximately 0.5% of the

Table 4.3. Average prevalence of major phyla and classes on high forage and high concentrate diets by time period

	Forage						Concentrate						
	Period 1		Period 2		Period 3		Period 1		Period 2		Period 3		SEM
Microbial Level	+	-	+	-	+	-	+	-	+	-	+	-	
Phylum Actinobacteria	3.05	3.15	2.28	2.30	2.37	2.47	3.17	3.25	3.16	2.82	2.03	2.58	0.215
Class Actinobacteria	3.05	3.15	2.28	2.30	2.37	2.47	3.17	3.25	3.16	2.82	2.03	2.58	0.215
Phylum Bacteroidetes	0.78	0.33	0.29	0.64	0.63	0.69	0.79	0.34	0.31	0.32	0.82	0.73	0.109
Class Bacteroidetes	0.54	0.051	0.21	0.29	0.29	0.38	0.52	0.053	0.053	0.050	0.49	0.42	0.097
Class Sphingobacteria	0.044	0.051	0.059	0.053	0.060	0.060	0.049	0.044	0.053	0.064	0.063	0.056	0.003
Class Flavobacteria	0.062	0.094	0.10	0.12	0.11	0.092	0.089	0.097	0.062	0.078	0.099	0.10	0.009
Phylum Firmicutes	87.06	87.68	87.97	86.29	86.37	86.72	87.29	86.93	88.53	88.82	86.56	87.00	0.412
Class Bacilli	2.66	2.79	2.77	2.16	2.33	1.66	2.87	1.98	2.18	2.22	2.84	2.94	0.208
Class Erysipelotrichi	0.44	1.12	1.8	1.29	1.29	1.21	1.06	1.04	0.45	1.28	1.32	1.23	0.186
Class Clostridia	83.89	83.69	83.33	82.76	82.68	83.77	83.28	83.85	85.86	85.26	82.33	82.76	0.524
Phylum Proteobacteria	4.94	4.71	4.69	5.74	5.90	5.35	4.78	5.20	3.77	4.06	5.62	5.19	0.322
Class Alphaproteobacteria	0.31	0.30	0.44	0.61	0.64	0.65	0.40	0.46	0.48	0.37	0.50	0.44	0.059
Class Deltaproteobacteria	0.44	0.43	0.46	0.54	0.50	0.51	0.41	0.44	0.44	0.41	0.39	0.47	0.022
Class Epsilonproteobacteria	0.29	0.28	0.31	0.35	0.33	0.33	0.27	0.29	0.29	0.27	0.35	0.31	0.014
Class Gammaproteobacteria	1.87	1.76	1.33	2.02	2.16	1.83	1.79	1.94	1.07	1.23	1.99	1.94	0.172
Class Betaproteobacteria	2.02	1.93	2.12	2.20	2.27	2.02	1.90	2.05	1.50	1.78	2.23	2.01	0.106
Phylum Verrucomicrobia	3.52	3.43	3.75	4.25	3.93	3.98	3.26	3.55	3.54	3.26	4.17	3.74	0.165
Class Verrucomicrobiae	3.52	3.43	3.75	4.25	3.93	3.98	3.26	3.55	3.54	3.26	4.17	3.74	0.165

Forage: Period 1: Control (Wk1); Period 2: Low CH_4 (Wks 3, 4, 6); Period 3: CH_4 back to baseline (Wks 10, 12); **Concentrate:** Period 1: Control (Wk 1); Period 2: Low CH_4 (Wks 3,4); Period 3: CH_4 back to baseline (Wks 6, 10, 12); + diet with monensin; - diet without monensin

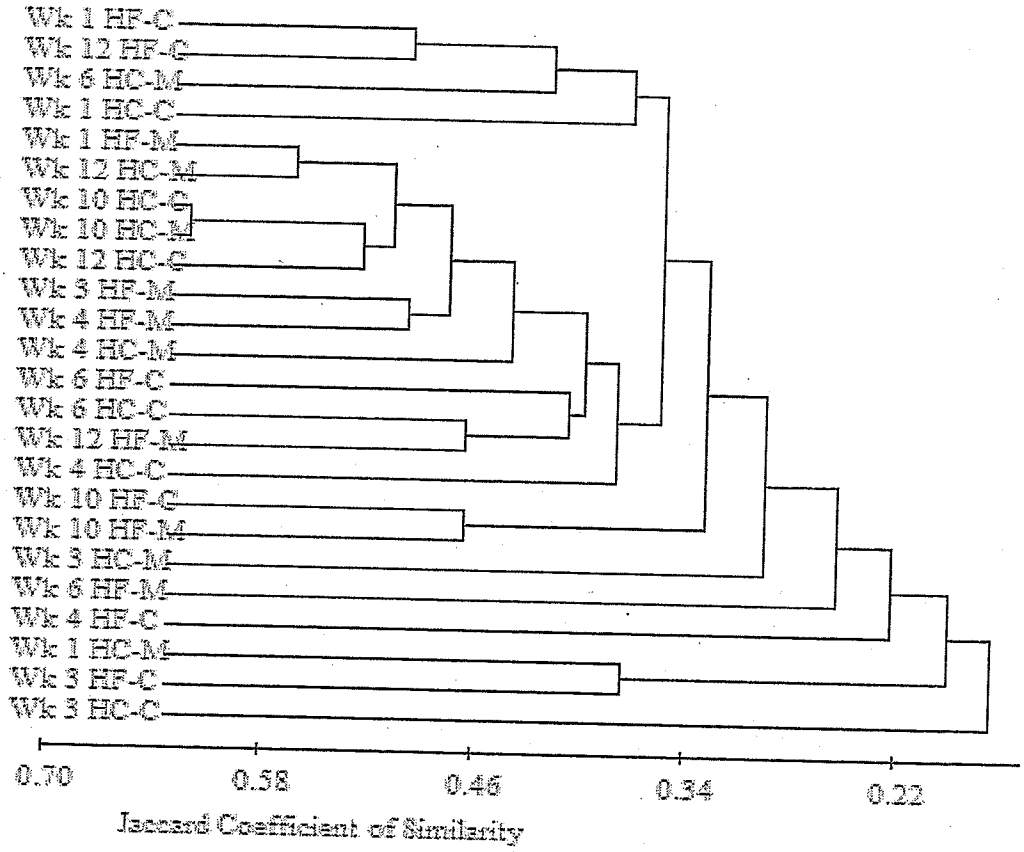

Figure 4.2: Cluster analysis of rumen methanogenic archaea grouped by diet (Diets - HF-C: High forage control; HF-M: High forage monensin; HC-C: High concentrate control; HC-M: High concentrate monensin). Scale value multiplied by 100 is the percentage of similarity.

total prevalence for both forage and concentrate diets at the phylum level. There were no statistical differences between all dietary treatments (concentrate versus forage or monensin versus control). In addition, there was no statistical difference between bacterial population prevalence over time.

4.4.3 Clustan Analysis

Multivariate analysis indicated that there were no clustering patterns that could group methanogens by monensin inclusion (Fig. 4.2). Methanogens appeared to be unaffected by the inclusion of monensin, however there is some clustering (approximately 60% similarity) occurring between diets (forage versus concentrate) which we can expect to see.

4.4.4 Real Time Polymerase Chain Reaction

In the forage plus monensin diet (Fig 4.3) there was a decrease in *Butyrivibrio fibrosolvens*, *Lactobacillus spp.*, *Ruminococcus flavefaciens*, *Selenomonas ruminatium*, but not *R. albus* when compared to the unsupplemented diet, and the decline in *Streptococcus bovis* was not substantial. The gram-positives *B. fibrosolvens, Lactobacillus spp.*, *R. albus*, *R. flavefaciens*, *S. ruminantium* and *S. bovis* decreased in abundance in the concentrate diet when monensin was included as compared to the unsupplemented diet (Fig 4.5). In both the forage and concentrate diets the gram-negative *Prevotella ruminicola* considerably increased in abundance with monensin supplementation (Figs 4.3 and 4.5).

In the forage diet there appeared to be little adaptation to monensin by gram-positive bacteria, with *R. flavefaciens* being the only species that showed adaptation as its values in period 3 significantly increased ($P < 0.05$) compared to period 2 (Fig 4.3). But

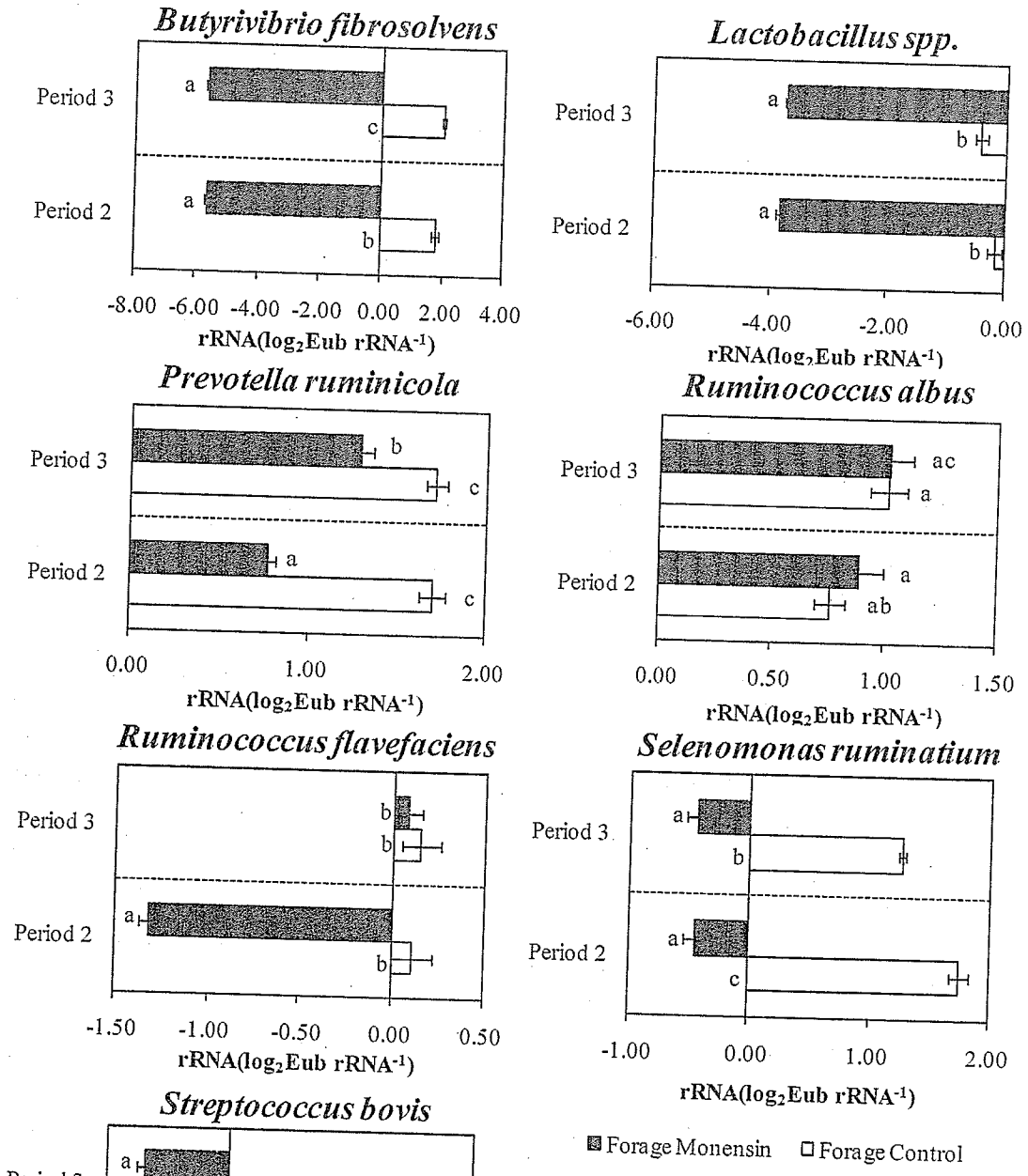

Figure 4.3: Relative abundance of specific taxonomic groups on the forage diet by time period [averaged values of allotted weeks (period 2 - low CH_4 represents wks 3, 4 & 6; period 3 – CH_4 back to baseline represents wks 10 & 12)]. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to the values in week one. Same lower case letters denote significant similarity and different letter denote significant difference between all periods and treatments.

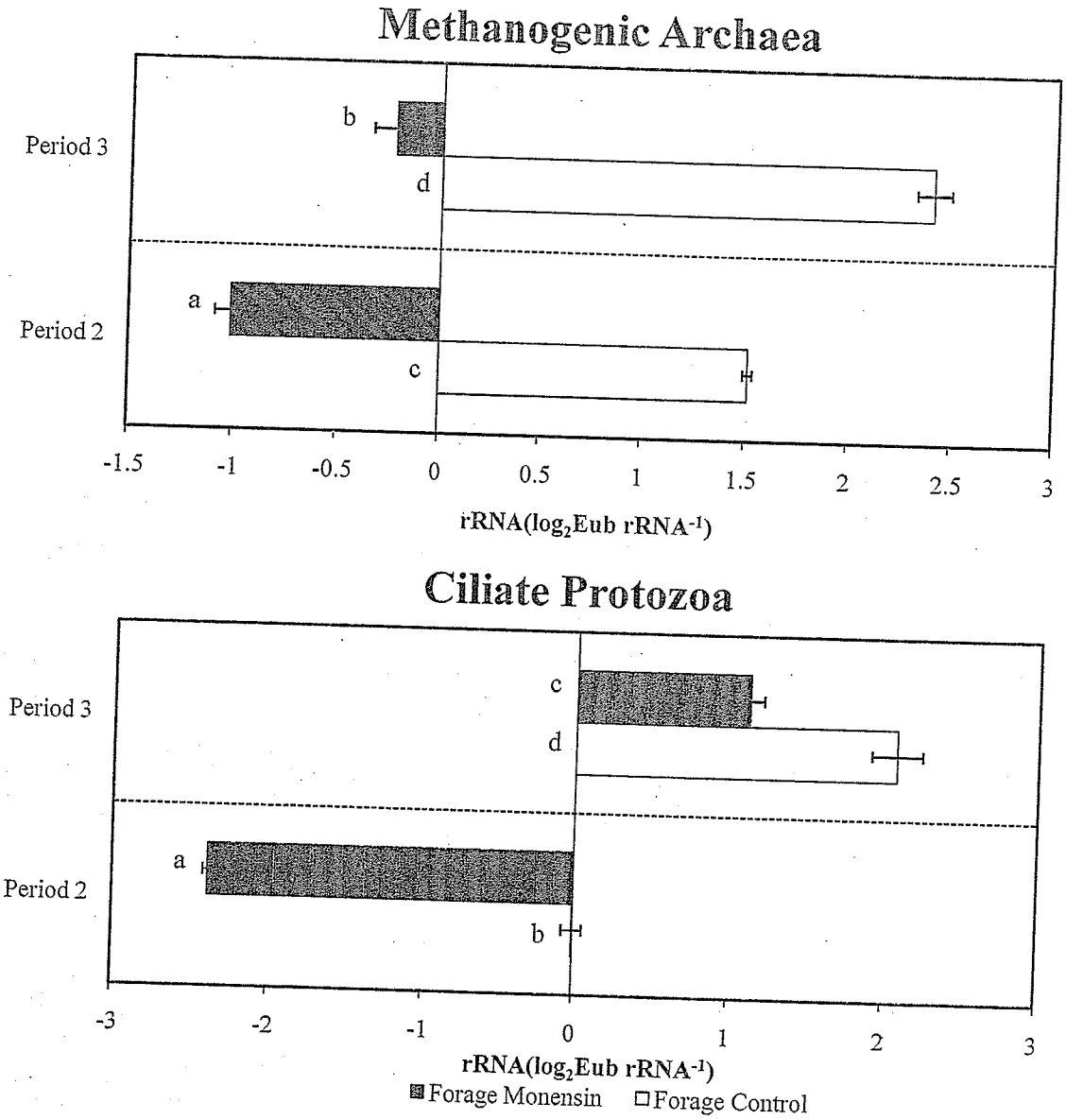

Figure 4.4: Relative abundance of methanogenic archaea and ciliate protozoa on the forage diet by time period [averaged values of allotted weeks (period 2 - low CH_4 represents wks 3, 4 & 6; period 3 – CH_4 back to baseline represents wks 10 & 12)]. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to the values in week one. Same lower case letters denote significant similarity and different letter denote significant difference between all periods and treatments.

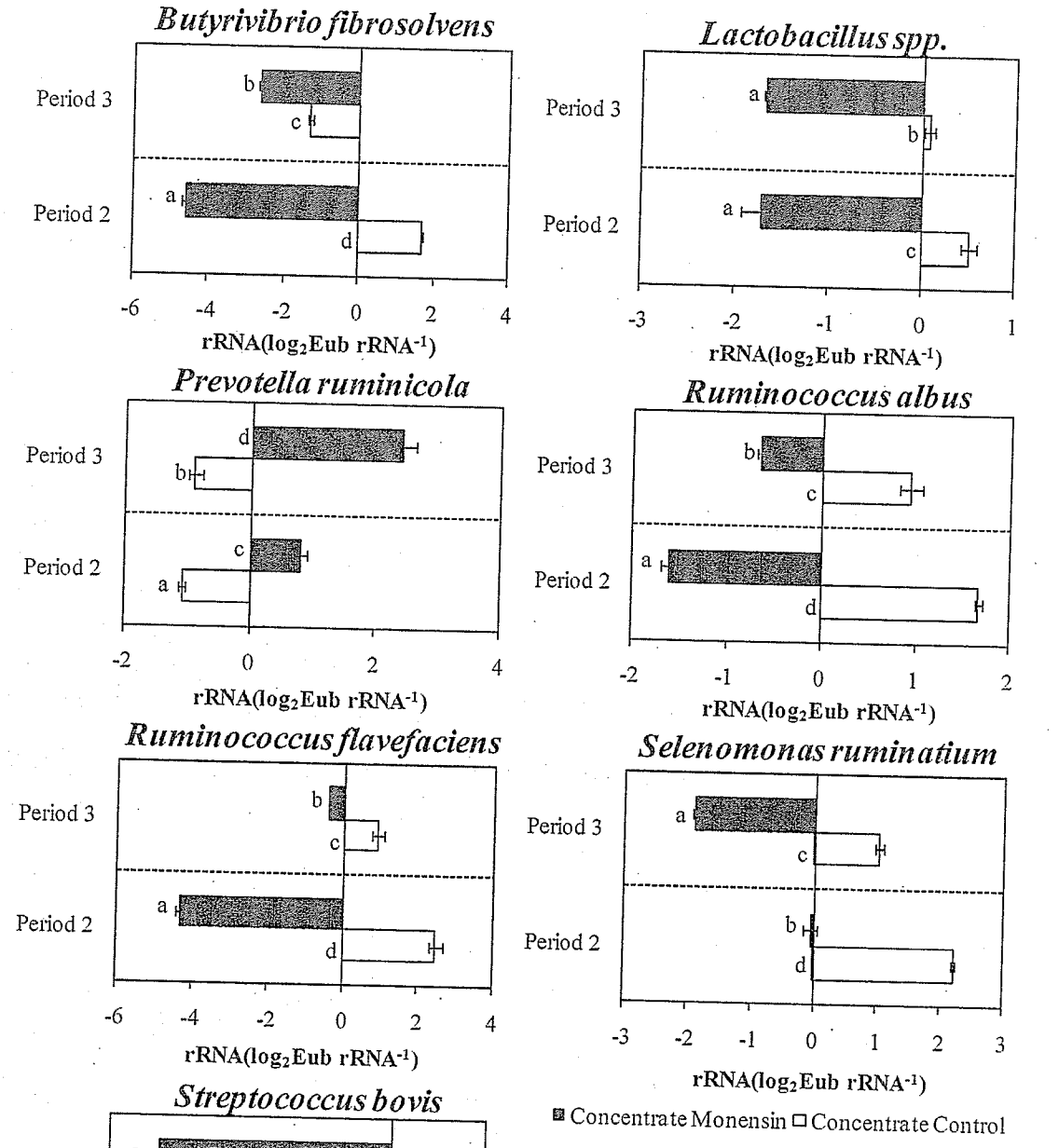

Figure 4.5: Relative abundance of specific taxonomic groups on the concentrate diet by time period [averaged values of allotted weeks (period 2 - low CH_4 represents wks 3 & 4; period 3 – CH_4 back to baseline represents wks 6, 10 & 12)]. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to the values in week one. Same lower case letters denote significant similarity and different letter denote significant difference between all periods and treatments.

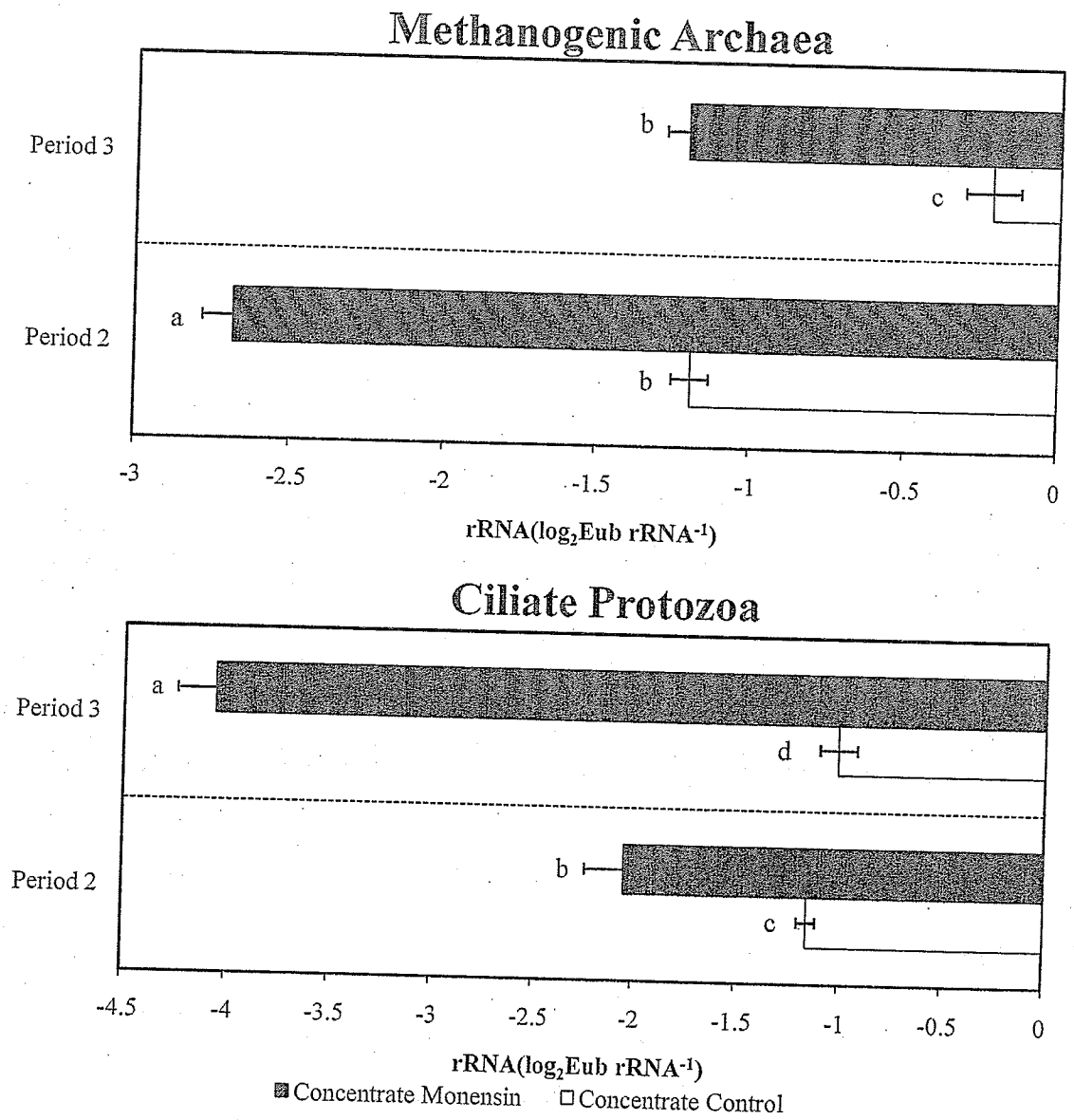

Figure 4.6: Relative abundance of specific taxonomic groups on the concentrate diet by time period [averaged values of allotted weeks (period 2 - low CH_4 represents wks 3 & 4; period 3 – CH_4 back to baseline represents wks 6, 10 & 12)]. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to the values in week one. Same lower case letters denote significant similarity and different letter denote significant difference between all periods and treatments.

the methanogenic archaea and ciliate protozoa did increase in abundance ($P < 0.05$) after an initial decrease while on forages (Fig 4.4). The concentrate diet showed *B. fibrosolvens*, *R. albus*, and *R. flavefaciens* adapted to the monensin treatment with their period 2 levels being significantly ($P < 0.05$) higher in period 3 versus period 2 (Fig 4.5). The methanogenic archeaea on the concentrate diet were initially decreased but then significantly ($P < 0.05$) increased in abundance over time, whereas the ciliate protozoa remained consistently lower than baseline levels (Fig 4.6).

4.5 DISCUSSION

To determine any temperature effect on methane levels a correlation was measured between the factors (methane levels for control and monensin treatments and temperature) for both the forage (Fig 4.1A) and concentrate (Fig 4.1B) diets. The correlations for all comparisons were relatively low suggesting the effect of temperature on methane emissions was not major. The interactions between methane and temperature needed to be considered as it may be an alternative explanation for adaptation in the rumen however from this data alone it is impossible to say whether temperature alone is responsible for the decrease in methane levels.

When TRFLP analysis was conducted over a 12 wk period (Fig. 1) the most prevalent phylum was the Firmicutes which accounted for over 80% of the population in both the forage (Fig. 1A) and grain diets (Fig. 1B). The other major phyla were the Proteobacteria (~5%), Verrucomicrobia (~3.5%), Actinobacteria (~2.5%), and Bacteroidetes (~0.5%). These proportions were approximately the same irrespective of

whether a forage or grain diet was consumed and demonstrate a remarkable level of stability at the phylum and the class level. However the inclusion of monensin had little influence on phylum structure of the prokarya (Table 4.3) or archaea (Fig. 4.2).

Using the search term "rumen" in the RDP (Cole et al. 2008) a distribution of all rumen sequences could be obtained. This analysis indicated that 71.2% of rumen sequences were from the Firmicutes, 18.1% from the Bacteroidetes, 7.1% from the Proteobacteria, 0.43% from the Actinobacteria, and approximately 1% from the Verrucomicrobia. We can thus conclude that the phylum prevalence we observed using TRFLP without 16S rDNA sequencing provides a representative distribution that is typical of the rumen ecosystem. We have previously demonstrated (Sepehri et al. 2007) that using TRFLP in conjunction with our described bioinformatic analytical pipeline provides robust data to the family level.

An interesting observation from both the data presented here and the RDP search was that the Verrucomicrobia are one of the major phyla in the rumen (Fig 4.1). In the RDP there are a large number of sequences within the Verrucomicrobia that are rumen affiliates, but none of them have been isolated. The closest affiliated isolate from the mammalian digestive tract is *Akkermansia muciniphila* a mucin degrading bacterium isolated from the human gut (Derrien et al. 2004). It is unclear what role mucin degradation per se would have, but the rumen is a plant polysaccharde rich environment containing structures with similarity to mucin carbohydrate moieties (Goldstein 2002; Flint et al. 2008). *A. muciniphilia* could consequently have a role in plant polysaccharide degradation (Flint et al. 2008).

It has been demonstrated in anaerobic sediments that the Verrucomicrobia are involved in anaerobic methane oxidation (Dunfield et al. 2007). In this syntrophic process oxygen or sulfate acts as the terminal electron acceptor (Raghoebarsing et al. 2006; Muyzer and Stams 2008). The rumen is an anaerobic environment and sulfate is almost always present in the rumen (McSweeny and Denman 2007; Spears 2003). We speculate that methane oxidation with sulfate as the terminal electron acceptor occurs in the rumen. To our knowledge this is a virtually unexplored process in rumen methanogenesis and future research should attempt isolation of members of the Verrucomicrobia from the rumen.

Monensin has been suggested as a means by which methane production in the rumen can be reduced but it is debatable whether the effects on methane are short or long term (Callaway et al. 2003). Poos et al. (1979), Perry et al. (1983), Rumpler et al. (1986), and Lana et al. (1997) demonstrated that the beneficial effects of monensin were long lasting but they did not measure methane emissions. Guan et al. (2006) observed that methane emissions returned to baseline levels within four to six weeks depending on carbon source. Beauchimin and McGinn (2005) fed cattle through various phases of production typically of a feedlot operation. Animals consumed concentrate diets supplemented with monensin and methane emissions were determined (Beauchemin and McGinn 2005). Although they did not specifically measure adaptation to monensin one interpretation of their data is that methane declined as animals became older, suggesting adaptation. In a similar study by McGinn et al. (2004) methane emissions declined when monensin supplemented animals were compared to controls but the experimental period was only 21 d.

We were able to demonstrate adaptation of some rumen species to monensin when the samples from Guan et al. (2006) were analyzed with real-time PCR. Real-time PCR data provided abundance data, while TRFLP provides prevalence data. Although there were no significant differences between treatments based on prevalence measures of prokaryotes (Table 4.3), or archaea (Fig. 4.2) there were clear differences in the abundances of species we hypothesized to be important.

In general, the major effects of monensin were on gram-positive hydrogen producing species (*Ruminococcus albus R. flavefaciens, Butyrivibrio fibrisolvens, Selenomonas ruminantium*) (Figs 4.3 and 4.5). The increase in *P. ruminicola*, a gram-negative is most likely the result of decreased competition for substrate with *S. bovis*. On the forage-monensin diet all gram-positive bacteria except *R. albus* decreased in abundance relative to the control. The differential response between *R. albus* and *R. flavefaciens* is not clear but this result has also been reported by other investigators (Weimer et al. 2008). It is possibly a consequence of access of monensin to the bacteria that cover the plant cell wall surface and we speculate that *R. albus* is protected in the biofilm on the plant surface.

On the concentrate-monensin diet all of the gram-positive hydrogen producing species decreased in abundance compared to the control. The adaptation observed in the gram-positive *R. albus*, *R. flavefaciens* and *B. fibrosolvens* and not any other species may be attributed to competitive advantage in the rumen. *B. fibrosolvens* can flourish in high grain diets as they are strongly amylolytic (Stewart et al. 1997). Whereas *R. albus* and *R. flavefaciens* can associate with the highly abundant *P. ruminicola* on plant surfaces, as the concentrate diet included 22.7% alfalfa silage, therefore taking advantage of the

protection given by the biofilm community and replenishing their populations (Stevenson and Weimer, 2007; Larue et al. 2005; Guan et al. 2006). Furthermore the rate of rumen turnover is faster on the high concentrate diet, and this may have affected the growth of the bacteria.

Although there was a negative effect on *Streptococcus bovis* it is not considered a hydrogen producing species but an active proteolytic organism. As *S. bovis* declined there was an increase in the gram-negative proteolytic species *Prevotella ruminicola*. We can consequently conclude that the increase in *P. ruminicola* is because of reduced competition for protein between *S. bovis* and *P. ruminicola*. Other investigators have discussed the role of monensin in reducing wasteful protein fermentation in the rumen and reduced competition for protein is one of these mechanisms (Houssein et al. 1991; Bach et al. 2005; Chen and Russell 1989).

Monensin effects are also observed in the methanogenic archaea (Figs. 4.4 and 4.6), whether this effect of monensin is direct or indirect is debatable as both have been reported to occur. Mathison et al. (1998) reported methanogens to adapt to monensin over time, where as other studies have shown no effect of monensin on methanogenic archaea (Weimer et al. 2008; Van Nevel and Demeyer 1977). The interaction of rumen methanogens with ciliate protozoa can also be explored as an explanation for the decrease in archaeal abundance (Tokura et al. 1999). Methanogens are known to associate with rumen protozoa (Finlay et al. 1994), and the decrease in monensin susceptible ciliate protozoa can correlate with the reduction in methanogens (Figs 4.4 and 4.6).

The adaptation trend seen in the methanogenic archaea on the forage and concentrate diets can potentially be explained in relation to the protozoa. A forage diet

has a more neutral pH which is favorable for protozoa growth, as well forage provides more surface area for growth in the rumen (Cheeke, 2005), thus protozoa abundance was able to increase and provide growing space for the monensin resistant methanogens. The methanogenic archaea increased in abundance on the concentrate as well however the protozoa did not. This is because the monensin susceptible protozoa could not regain population due to a lower pH in the rumen, it has been reported that cattle fed a high concentrate diet and a rumen pH below 6.0 were defaunated of protozoa over time. (Nagaraja and Titgemeyer 2007; Franzolin and Dehority 1996). The rise in archaeal abundance can thus be attributed to a resistance to monensin and an increase of available hydrogen, and their initial decline in both diets is a result of the decreased protozoa.

We conclude that providing an animal trial is at least six wk in length some rumen microbial adaptation to monensin occurs (Fig. 4.7). Although it is not possible to unequivocally concluded that the effect of monensin is on hydrogen producing gram-positive bacteria it is appealing given the correlation of methanogens and methane within a relatively constrained time period. A striking feature of the adaptation is that it occurs more quickly with high concentrate diets than with forage diets. We attribute this to the fact that the turnover, and consequently growth rate of bacteria, is slower in forage diets than in concentrate diets (Sniffen et al. 1992; Fox et al. 1992).

4.6 ACKNOWLEDGMENTS

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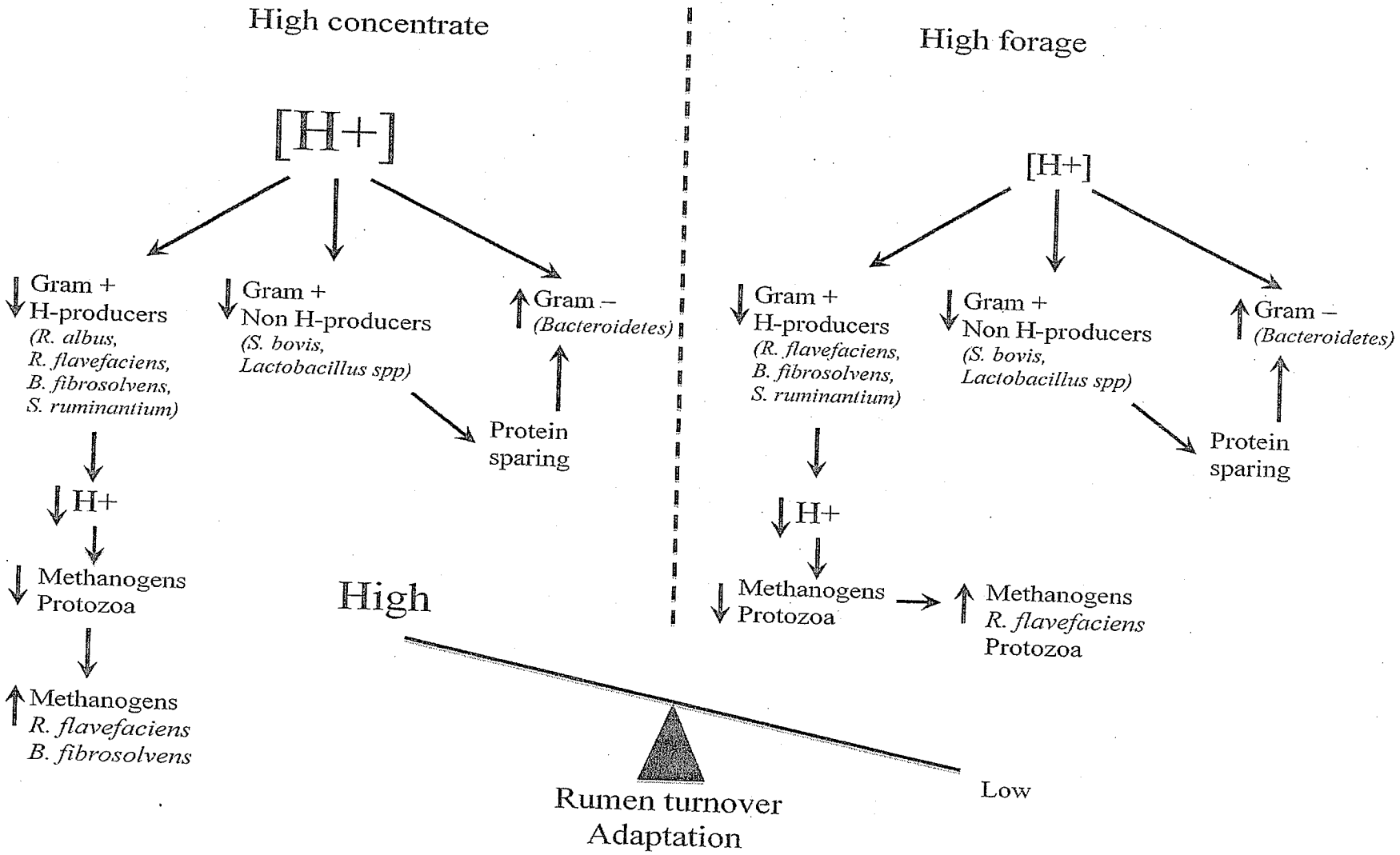

Figure 4.7: Summary of research

5.0 GENERAL SUMMARY

Methane emissions from ruminant livestock have a dominant impact on the agriculture greenhouse gas inventory and in order to see some relief a mitigation strategy must be developed. Ionophores have been shown to reduce methane emissions from ruminants (Van Vugt et al. 2005) among many other beneficial effects such as reduced bloat, increased feed efficiency, and decrease lactic acid production to help prevent rumen acidosis (Tedeschi et al. 2003), and therefore could provide an avenue for better overall ruminant production.

Ionophores, such as monensin, have antibiotic properties mainly against gram-positive bacteria (Callaway et al. 2005). In the rumen many gram-positive bacteria are also hydrogen producing bacteria (Russell and Rychlik 2001), which connects them to methanogenesis. Rumen methanogenesis requires hydrogen to be produced by bacteria during fermentation to convert carbon dioxide to methane (Gottschalk 1988). Using monensin will cause a decrease in the populations of gram-positive bacteria and consequently hydrogen (Russell and Strobel 1989) and without hydrogen methanogenesis cannot occur and methane emissions will decline.

Previous research by Guan et al. (2006) showed monensin to successfully decrease methane emissions from feed lot cattle, however after only a few weeks methane emissions returned to baseline levels. In this study, rumen fluid samples taken from Guan et al. (2006) were analyzed to examine rumen microbial ecology and the possible adaptation to monensin. It was found using TRFLP that monensin did not affect the prevalence of bacteria at the phylum level, however it did indicate what major phyla

were at play and gave a sense of where to proceed with RT-PCR. The TRFLP prevalence data identified the Verrucomicrobia as a major phylum (~3.5%) of the rumen, however very little is known about them. Verrucomicrobia are known to be anaerobic methane oxidizers in thermophilic and acidophilic environments, which prompted a question in their role in rumen fermentation. Further research into their ecology may reveal them to play an integral part in methane reduction from the rumen. TRFLP using primers specific to methanogens and clustering software demonstrated the methanogens were not affected by the monensin treatment.

The abundance data obtained from RT-PCR showed monensin caused most of the gram-positive hydrogen producers to decrease in both diets and adaptation was observed in *B. fibrosolvens*, *R. albus* and *R. flavefaciens* on the concentrate diet and only in *R. flavefaciens* on the forage diet. These adaptations may be explained by protective microbial community interactions in the rumen as well as for the amylolytic *B. fibrosolvens* affinity for high grain diets. The gram-negative species, *P. ruminicola* showed a considerable increase compared to baseline over time, which can be attributed to the lack of nutrient competition in the rumen.

The methanogenic archaea and ciliate protozoa were also affected by the monensin treatment. The response of the archaea can be associated with the response of the protozoa. Many methanogenic species live symbiotically with protozoa in the rumen (Finlay et al. 1994), and the decrease of protozoa in the rumen because of monensin would cause a decrease in methanogenic populations. Therefore the effect of monensin on archaea is indirect whereas the effect on protozoa is direct.

To consider the effects of temperature on methane levels a correlation was measured. These correlations could not eliminate the influence of temperature however they were low enough to conclude it is unlikely the influence is large.

A significant difference was in the type of carbohydrate (forage versus concentrate) fed in the diet. The methane emissions for those animals on the concentrate diet returned to baseline levels two weeks earlier than those fed the forage diet. This is attributed to the fact that the rate of rumen turnover is higher in concentrate diet (Sniffen et al. 1992; Fox et al. 1992).

Results from this study are helpful in showing adaptation to monensin can occur in the rumen. Also the observed diet effect correlates the monensin adaptation to rumen turnover. And now research efforts can focus on using monensin as a continuous tool for methane reduction or research on other methods for long term methane suppression can be investigated.

6.0 CONCLUSIONS

It can be concluded that:

- Adaptation of some bacteria to monensin did occur in the rumen on both forage and concentrate diets.
- There was a carbohydrate (forage versus concentrate) effect on rate of adaptation, with the concentrate diet showing adaptation two weeks earlier than the forage diet and this can be attributed to rumen turnover being characteristically faster with a concentrate diet.
- The phylum Verrucomicrbia is among the most represented in the rumen however there is very little known about it. In other environments the Verrucomicrobia have been found to be methane oxidizers, therefore further studies should be performed to investigate its role in the rumen.
- Methanogenic populations were not directly affected by the monensin treatment on the forage or concentrate diet. Their abundance initially decreased on both diets however this is a result of a large number of archaea known to reside on monensin susceptible protozoa.
- The ciliate protozoa populations were affected by monensin on both the forage and concentrate diet. Their abundance did return in the forage diet due to increased surface area and a near neutral pH, but not in the concentrate diet where the pH was more acidic.

- Temperature had low correlation with methane levels and therefore appears to have little influence.
- Further research must be conducted to completely understand the adaptation process.

7.0 REFERENCES

1. **Applied Biosystems. 2001.** User Bulletin #2: ABI 7700 Sequence Detection System. [Online] Available: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf

2. **Bach, A., Calsamiglia, S. and Stern, M. D. 2005.** Nitrogen Metabolism in the Rumen. J. Dairy Sci. **88:** E9-E21.

3. **Badawy, S. A., Younis, M., Shalash, M. R., Nawito, M. F., Mansour, S. A. and Rakha. G. 1993.** Monensin Effects on Rumen Metabolic Profile, Methane Production, and Protozoal Population in Buffalo-heifers. Egypt J. Vet. Sci. **30:** 49-56.

4. **Beauchemin, K. A. and McGinn, S. M. 2005.** Methane emissions from feedlot cattle fed barley or corn diets. J. Anim. Sci. **83:** 653-661.

5. **Beauchemin, K. A. and McGinn, S. M. 2006.** Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. J. Anim. Sci. **84:** 1489-1496.

6. **Bergen, W. G. and Bates, D. B. 1984.** Ionophores: Their Effect on Production Efficiency and Mode of Action. J. Anim. Sci. **58:** 1465-1483.

7. **Bergey DH. 1994.** The methanogens. p. 719-725 *In*: Bergey's Manual of Determinative Bacteriology Edited by Holt JG, 9 ed. Baltimore: Williams and Wilkins Co.

8. **Bhandari, S. K., Nyachoti, C. M. and Krause, D. O. 2008.** Raw potato starch in weaned pig diets and its influence on post-weaning scours and the molecular microbial ecology of the digestive tract. J. Anim. Sci. [Epub ahead of print]

9. **Boadi, D. A., Wittenberg, K. M. and Kennedy, A. D. 2002.** Validation of the sulphur hexafluoride (SF6) tracer gas technique for measurement of methane and carbon dioxide production by cattle. Can. J. Anim. Sci. **82:** 125-131.

10. **Callaway, T. R. and Martin, S. A. 1996.** Effects of Cellobiose and Monensin on In Vitro Fermentation of Organic Acids be Mixed Ruminal Bacteria. J. Dairy Sci. **80:** 1126-1135.

11. **Callaway, T. R., Adams, K. A. and Russell, J. B. 1999.** The Ability of "Low G + C Gram-Positive" Ruminal Bacteria to Resist Monensin and Counteract Potassium Depletion. Curr. Microbiol. **39:** 226-230.

12. **Callaway, T. R., Edrington, T. S., Rychlik, J. L., Genovese, K. J., Poole, T. L., Jung, Y. S., Bischoff, K. M., Anderson, R. C. and Nisbet, D. J. 2003.** Ionophores: Their Use as Ruminant Growth Promotants and Impact on Food Safety. Curr. Issues Intest. Microbiol. **4:** 43-51.

13. **Callaway, T. R., Martin, S. A., Anderson, R. C., Edrington, T. S., Nisbet, D. J. and Genovese, K. J. 2005.** Rumen Microbiology, p. 773-776. *In*: Encyclopedia of Animal Science, Marcel Dekker, Inc.

14. **Chaban, B., Ng, S. Y. M. and Jarrell, K. F. 2006.** Archaeal habitats — from the extreme to the ordinary. Can. J. Microbiol. **52:** 73–116.

15. **Cheeke, P. R. 2005.** Applied Animal Nutrition, Feeds and Feeding 3rd Ed. Pearson Prentice Hall, USA.

16. **Chen, G. and Russell, J. B. 1989.** More Monensin-Sensitive, Ammonia-Producing Bacteria from the Rumen. Appl. Environ. Microbiol. **55:** 1052-1057.

17. **Chen, M. and Wolin, M. J. 1979.** Effect of Monensin and Lasalocid-Sodium on the Growth of Methanogenic and Rumen Saccharolytic Bacteria. Appl. Environ. Microbiol. **38:** 72-77.

18. **Chiba, L. I. 2007.** Rumen Microbiology and Fermentation, p. 54 – 73 *In*: Animal Nutrition Handbook. [Online.] http://www.ag.auburn.edu/~chibale/animalnutrition.html

19. **Chow, J. M. and Russell, J. B. 1990.** Effect of Ionophores and pH on Growth of Streptococcus bovis in Batch and Continuous Culture. Appl. Environ. Microbiol. **56:** 1588-1593.

20. **Christophersen, C. T., Wright, A. –D. G. and Vercoe, P. E. 2004.** Examining diversity of free-living methanogens and those associated with protozoa in the rumen. J. Anim. Feed Sci. **13**(Suppl. 1): 51-54.

21. **Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M. and Tiedje, J. M. 2008.** The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. Advance Access November 2008. doi:10.1093/nar/gkn879.

22. **Coleman, G. S. 1980.** Rumen Ciliate Protozoa. Adv. in Parasitol. **18:** 121-173.

23. **Denman, S. E. and McSweeney, C. S. 2006.** Development of a real-timePCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol. Ecol. **58:** 572–582.

24. **Dennis, S. M. and Nagaraja, T. G. 1981.** Effect of Lasalocid or Monensin on Lactate-Producing os Using Rumen Bacteria. J. Anim. Sci. **52:** 418-426.

25. **Dennis, S. M. and Nagaraja, T. G. 1986.** Effect of lasalocid, monensin and thiopeptin on rumen protozoa. Res. Vet. Sci. **41:** 251-256.

26. **Deppenmeier, U. 2002.** The Unique Biochemistry of Methanogenesis. Prog. Nucleic Acid Res. Mol. Biol. **71:** 223-282.

27. **Derrien, M., Vaughan, E. E., Plugge, C. M. and de Vos, W. M. 2004.** Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. Microbiol. **54:** 1469-1476.

28. **Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B., Hou, S., Ly, B., Saw, J. H., Zhou, Z., Ren, Y., Wang, J., Mountain, B. W., Crowe, M. A., Weatherby, T. M., Bodelier, P. L. E., Liesack, W., Feng, L. Wang, L. and Alam, M. 2007.** Methane oxidation by an extremely acidophilic bacterium of the phylum Verruocomicrobia. Nature. **450:** 879-882.

29. **Edrington, T. S., Callaway, T. R., Varey, P. D., Jung, Y. S., Bischoff, K. M., Elder, R. O., Anderson, R. C. and Nisbet, D. J. 2003.** Effect of feeding the ionophores monensin and laidlomycin propionate and the antimicrobial bamnbermycin to sheep experimentally infected with *E. coli* O157:H7 and *Salmonella* Typhimurium. J. Anim. Sci. **81:** 553-560.

30. **Environment Canada. 2008.** Canada's 2006 Greenhouse Gas Inventory. [Online] Available: http://www.ec.gc.ca/pdb/ghg/inventory_report/2006/som-sum_eng.cfm.

31. **Europa. 2005.** Ban on antibiotics as growth promoters in animal feed enters into effect. [Online] Available: http://europa.eu/rapid/pressReleasesAction.do?reference=IP/05/1687&format=HTML&aged=0&language=EN&guiLanguage=en.

32. **Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M. and Hirt, R. P. 1994.** Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. **117:** 157-162.

33. **Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R. and White, B. A. 2008.** Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat. Rev. Microbiol. **6:** 121-131.

34. **Fox, D. G., Sniffen, C. J., O'Connor, J. D., Russell, J. B. and Van Soest, P. J. 1992.** A Net Carbohydrate and Protein System for Evaluating Cattle Diets: III. Cattle Requirements and Diet Adequacy. J. Anim. Sci. **70:** 3578-3596.

35. **Franzolin, R. and Dehority, B. A. 1996.** Effect of Prolonged High-Concentrate Feeding on Ruminal Protozoa Concentrations. J Anim Sci **74:** 2803-2809.

36. **Galindo, J., Elias, A., Palenzuela, T., Pérez, M. C. and Aldama, A. I. 2003.** Effect of monensin on the *in vitro* methane production in three ruminal ecological systems. Cuban J. Agric. Sci. **37:** 181-186.

37. **Garrett, R. H. and Grisham, C. M. 1999.** Biochemistry, Second Edition. Second edition. Brooks/Cole, Thomson Learning Inc., Pacific Grove, CA, USA

38. **Garrity, G. M., Lilburn, T. G., Cole, J. R., Harrison, S. H., Euzéby, J. and Tindall, B. J. 2007.** Taxonomic Outline of the Bacteria and Archaea: Part 1: The "*Archaea*", Phyla "*Crenarchaeota*" and "*Euryarchaeota*". Michigan State University Board of Trustees, Michigan USA.

39. **Goldstein, I. J. 2002.** Lectin Structure–Activity: The Story Is Never Over. J. Agric. Food Chem. **50:** 6583-6585.

40. **Gottschalk, G. 1988.** Bacterial Metabolism, Second Edition, Springer-Verlag New York Inc. 175 Fifth Avenue, New York, New York, 10010

41. **Guan, H., Wittenberg, K. M., Ominski, K. H. and Krause, D. O. 2006.** Efficacy of ionophores in cattle diets for mitigation of enteric methane. J. Anim. Sci. **84:** 1896-1906.

42. **Gyulai, F. and Baran. M. 1988.** Effect of monensin on rumen ciliate protozoa in sheep. Arch. Tierernahr. **38:** 153-157.

43. **Hino, T. 1981.** Action of monensin on rumen protozoa. Jpn. J. Zootech. Sci. **52:** 171-179.

44. **Hino, T. and Russell, J. B. 1987.** Relative contributions of ruminal bacteria and protozoa to the degradation of protein in vitro. J. Anim. Sci. **64:**261-270.

45. **Houssein, H. S., Stern, M. D. and Jordan, R. M. 1991.** Influence of dietary protein and carbohydrate sources on nitrogen metabolism and carbohydrate fermentation by ruminal microbes in continuous culture. J. Anim. Sci. **69:** 2123-2133.

46. **Hungate, R. E. 1966.** The Rumen and its Microbes. Academic Press Inc, 111 Fifth Avenue, New York, New York 10003

47. **Hungate, R. E., Dougherty, R. W., Bryant, M. P. and Cello, R. M. 1952.** Microbiological and physiological changes associated with acute indigestion in sheep. Cornell Vet. **42:** 423-449. 29.

48. **Ishler, V. A., Heinrichs, A. J., Varga, G. B., 1996.** From feed to milk: understanding rumen function. Extension circular/Penn State College of Agricultural Sciences 422, Pennsylvania State University, University Park, PA.

49. **Janssen, P. H., and Kirs, M. 2008.** Structure of the Archaeal Community of the Rumen. Appl. Environ. Microbiol. **74:** 3619-3625.

50. **Jarvis, G. N., Strömpl, C., Burgess, D. M., Skillman, L. C., Moore, E. R. B. and Joblin, K. N. 2000.** Isolation and Identification of Ruminal Methanogens from Grazing Cattle. Curr. Microbiol. **40:** 327-332.

51. **Johnson, K. A. and Johnson, D. E. 1995.** Methane Emissions from Cattle. J. Anim. Sci. **73:** 2483-2492.

52. **Jukes, T. H. 1972.** Antibiotics in Animal Feeds and Animal Production. Bioscience. **22:** 526-534.

53. **Kamra, D. N. 2005.** Rumen microbial ecosystem. Curr. Sci. **89:** 124-135.

54. **Kebreab, E., Clark, K., Wagner-Riddle, C. and France, J. 2006.** Methane and nitrous oxide emissions from Canadian animal agriculture: A review. Can. J. Anim. Sci. **86:** 135-158.

55. **Kent, A.D., Smith, D. J., Benson, B. J. and Triplett, E. W. 2003.** A web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl. Environ. Microbiol **69:** 6768-6776.

56. **Khafipour, E., Li, S. Plaizier, J. C. and Krause, D. O. 2008.** Rumen microbial community analysis using T-RFLP and real-time PCR in dairy cattle experimentally induced with subacute ruminal acidosis (SARA). (Submitted to Appl. Environ. Microbiol.)

57. **Lan, Y., Xun, S., Tamminga, S., Williams, B. A., Verstegen, M. W. A. and Erdi, G. 2004.** Real-Time PCR Detection of Lactic Acid Bacteria in Cecal Contents of Eimeria tenella-Infected Broilers Fed Soybean Oligosaccharides and Soluble Soybean Polysaccharides. Poult. Sci. **83:** 1696-1702.

58. **Lana, R. P., Fox, D. G., Russell, J. B. and Perry, T. C. 1997.** Influence of monensin on Holstein steersfed high-concentrate diets containing soybean meal or urea. J. Anim. Sci. **75:** 2571-2579.

59. **Lana, R. P. and Russell, J. B. 1996.** Use of Potassium Depletion to Assess Adaptation of Ruminal Bacteria to Ionophores. Appl. Environ. Microbiol. **62:** 4499-4503.

60. **Lane, D. J. 1991.** 16S/23S rRNA sequencing. p. 115-175. *In:* E. Stackebrandt and M. Goodfellow (Eds.), Nucleic acid techniques in bacterial systematics, John Wiley and Sons, New York, NY, USA.

61. **Larue, R., Yu, Z., Parisi, V. A., Egan, A. R. and Morrison, M. 2005.** Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and rrs gene sequencing. Environ. Microbiol. **7:** 530–543.

62. **Lewis, K., Naroditskaya, V. Ferrante, A. and Fokina, I. 1994.** Bacterial Resistance to Uncouplers. J. Bioenerg. Biomembr. **20:** 639-646

63. **Mackie, R. I. and Cann, K. O. 2005.** A Review of Gastrointestinal Microbiology with Special Emphasis on Molecular Ecology Approaches. p:175-198. *In* H. P. S. Makkar and G. J. Viljoen (eds.), Applications of Gene-Based Technologies for Improving Animal Production and Health in Developing Countries, Springer, Netherlands.

64. **Martinele, I., Eifert, E. C., Lana, R. P., Arcuri, P. B. and D'Agosto, M. 2008.** Effect of monensin and soybean oil on rumen ciliate protozoa and correlation between protozoa with ruminal fermentation and digestive parameters R. Bras. Zootec. **37:** 1129-1136.

65. **Mathison, G. W., Okine, E. K., McAllister, T. A., Dong, Y., Galbraith, J. and Dmytruk, O. I. N. 1998.** Reducing methane emissions from ruminant animals. J. Appl. Anim. Res. **14:** 1-28.

66. **McAllister, T. A. and Newbold, C. J. 2008.** Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agr. **48:** 7-13.

67. **McGinn, S. M., Beauchemin, K. A., Coates, T. and Colombatto, D. 2004.** Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast and fumaric acid. J. Anim. Sci. **82:** 3346-3356.

68. **McSweeney, C. S. and Denman, S. E. 2007.** Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. J. Appl. Microbiol. **103:** 1757-1765.

69. **Microbial Community Analysis III. 2007.** Department of Biological Science, University of Idaho [Online] Available: http://mica.ibest.uidaho.edu/.

70. **Moss, A. R., Jouany, J. P. and Newbold, J. 2000.** Methane production by ruminants: its contribution to global warming. Ann. Zootech. **49:** 231–253.

71. **Muyzer, G. and Stams, A. J. M. 2008.** The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. **6:** 441-454.

72. **Muyzer, G., De Waal, E. C. and Uitierlinden, A. G. 1993.** Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. Appl. Environ. Microbiol. **59:** 695-700.

73. **Nagaraja, T. G. and Titgemeyer, E. C. 2007.** Ruminal Acidosis in Beef Cattle: The Current Microbiological and Nutritional Outlook. J. Dairy Sci. **90:** E17-E38.

74. **Nannen, N. L. and Hutkins, R. W. 1990.** Intracellular pH Effects in Lactic Acid Bacteria. J. Dairy Sci. **74:** 741-746.

75. **Newbold, C. J., Lopez, S., Nelson, N., Ouda, J. O., Wallace, R. J. and Moss, A. R. 2005.** Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. Br. J. Nutr. **94:** 27-35.

76. **Newbold, C. J., Wallace, R. J. and Walker, N. D. 1993.** The effect of tetronasin and monensin on fermentation, microbial numbers and the development of ionophore-resistant bacteria in the rumen. J. Appl. Bacteriol. **75:** 129-134.

77. **Odongo, N. E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M. M., Hook, S. E., Gray, J. T., Kebreab, E., France, J. and McBride, B. W. 2007.** Long-Term Effects of Feeding Monensin on Methane Production in Lactating Dairy Cows. J. Dairy Sci. **90:** 1781–1788.

78. **Ohene-Adjei, S., McAllister, T., Teather, R. and Forster, R. 2007.** Plant extracts increase the diversity of methanogenic archaea in the ovine rumen. Environ. Microbiol., in press.

79. **Perry, T. W., Shields, D. R., Dunn, W. J. and Mohler, M. T. 1983.** Protein levels and monensin for growing and finishing steers. J. Anim. Sci. **57:** 1067-1076.

80. **Poos, M. I., Hanson, T. L. and Klopfenstein, T. J. 1979.** Monensin effects on diet digestibility, ruminal protein bypass, and microbial protein synthesis. J. Anim. Sci. **48:** 1516-1524.

81. **Prescott, L. M., Harley, J. P. and Klein, D. A. 2002.** Microbiology, Fifth Edition. The McGraw-Hill Companies Inc., New York, NY, USA.

82. **Pressman, B. C. 1976.** Biological Applications of Ionophores. Annu. Rev. Biochem. **45:** 501-530.

83. **Raghoebarsing, A. A., Pol, A., van de Pas-Schoonen, K. T., Smolders, A. J. P., Ettwig, K. F., Rijpstra, W. I. C, Schouten, S., Sinninghe Damste´, J. S., Op den Camp, H. J. M., Jetten, M. S. M and Strous, M. 2006.** A microbial consortium couples anaerobic methane oxidation to denitrification. Nature. **440:** 918-921.

84. **Richardson, L. F., Raun, A. P., Potter, E. L., Cooley, C. O. and Rathmacher, R. P. 1976.** Effect of monensin on rumen fermentation *in vitro* and *in vivo*. J. Anim. Sci. **43:** 657-664.

85. **Rodrigues, P. H. M., Mattos, W. R. S., Meyer, P. M., Lucci, C. S. and Melotti, L. 2004.** Effects of monensin level and roughage/concentrate ratio on ruminal fermentation in bovines. J. Anim. Feed Sci. **13:** 195-198.

86. **Rouviere, P. E., and Wolfe, R. S. 1988.** Novel biochemistry of methanogenesis. J. Biol. Chem. **263:** 7913-7916.

87. **Rumpler, W. V., Johnson, D. E. and Bates, D. B. 1986.** The effect of high dietary cation concentration on methanogenesis by steers fed diets with and without ionophores. J. Anim. Sci. **62:** 1737-1741.

88. **Russell, J. B. 1991.** Intracellular pH of Acid-Tolerant Ruminal Bacteria. Appl. Environ. Microbiol. **57:** 3383-3384.

89. **Russell, J. B. and Houlihan, A. J. 2003.** Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiol. Rev. **27:** 65-74.

90. **Russell, J. B. and Rychlik, J. L. 2001.** Factors that Alter Ruman Microbial Ecology. Science. **292:** 1119-1122.

91. **Russell, J. B. and Strobel, H. J. 1989.** Effects of Ionophores on Ruminal Fermentation. Appl.Environ. Microbiol. **55:** 1-6.

92. **Sepehri, S., Kotlowski, R., Bernstein, C. N. and Krause, D. O. 2007.** Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. Inflamm. Bowel Dis. **13:** 675-683.

93. **Singh, G. P. and Mohini, M. 1999.** Effect of Different Levels of Rumensin in Diet on Rumen Fermentation, Nutrient Digestibility and Methane Production in Cattle. Asian Australas. J. Anim. Sci. **12:** 1215-1221.

94. **Sniffen, C. J., O'Connor, J. D., Van Soest, P. J., Fox, D. G. and Russell, J. B. 1992.** A Net Carbohydrate and Protein System for Evaluating Cattle Diets: II. Carbohydrate and Protein Availability. J. Anim. Sci. **70:** 3562-3577.

95. **Spears, J. W. 2003.** Trace mineral bioavailability in ruminants. J. Nutr. **133:** 1506S-1509S.

96. **Stevenson, D. M. and Weimer, P. J. 2007.** Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. **75:** 165–174.

97. **Stewart, C. S., Flint, H. J. and Bryant M. P. 1997.** The Rumen Bacteria. Pages 10-55 in P. N. Hobson and C. S. Stewart ed. The Rumen Microbial Ecosystem, Second Edition. Blackie Academic & Professional, an imprint of Chapman & Hall, London, UK.

98. **Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M. and Firkins, J. L. 2004.** Development of an Assay to Quantify Rumen Ciliate Protozoal Biomass in Cows Using Real-Time PCR. J. Nutr. **134:** 3378-3384.

99. **Tedeschi, L. U., Fox, D. G. and Tylutki, T. P. 2003.** Potential Environmental Benefits of Ionophores in Ruminant Diets. J. Environ. Qual. **32:** 1591-1602.

100. **Thornton, J. H., and Owens, F. N. 1981.** Monensin supplementation and *in vivo* methane production by steers. J. Anim. Sci. **52:** 628-634.

101. **Tokura, M., Chagan, I., Ushida, K. and Kojima, Y. 1999.** Phylogenetic Study of Methanogens Associated with Rumen Ciliates. Curr. Microbiol. **39:** 123-128.

102. **Van Nevel, C. J., and Demeyer, D. I. 1977.** Effect of Monensin on Rumen Metabolism *in vitro*. Appl. Environ. Microbiol. **34:** 251-257.

103. **Van Vugt, S. J., Waghorn, G. C., Clark, D. A. and Woodward, S. L. 2005.** Impact of monensin on methane production and performance of cows fed forage diets. p. 362-366. *In*: vol 65. Proceedings of the New Zealand Society of Animal Production, New Zealand.

104. **Walter, J. C., Hertel, C. Tannock, G. W., Lis, C. M., Munro, K. and Hammes, W. P. 2001.** Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella Species in Human Feces by Using Group-Specific PCR Primers and Denaturing Gradient Gel Electrophoresis. Appl. Environ. Microbiol. **67:** 2578-2585.

105. **Wang R. F., Cao, W. W. and Cernigliaf, C. E. 1997.** PCR detection of *Ruminococcus* spp. in human and animal faecal samples. Mol. Cell Probes. **11:** 259-265.

106. **Weimer, P. J. 1998.** Manipulating Ruminal Fermentation: A Microbial Ecological Perspective. J. Anim. Sci. **76:** 3114-3122.

107. **Weimer, P. J., Stevenson, D. M., Mertens, D. R. and Thomas, E. E. 2008.** Effect of monensin feeding and withdrawal on populations of individual bacterial species in the rumen of lactating dairy cows fed high-starch rations. Appl. Microbiol. Biotechnol. **80:** 135-145.

108. **Whitford, M. F., Teather, R. M. and Forster, R. J. 2001.** Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiology, **1:** 5-10.

109. **Wittenberg, K. M. 2003.** Enteric methane emissions and mitigation opportunities for Canadian cattle production systems. p. 25-35. Proceedings of the 24th Western Nutrition Conference, Winnipeg, Manitoba.

110. **Woodward, S. L., Waghorn, G. C., Ulyatt, M. J. and Lassey, K. R. 2001.** Early indications that feeding *Lotus* will reduce methane emissions from ruminants. p. 23-26. *In*: vol: 61. Proceedings of the New Zealand Society of Animal Production, New Zealand.

111. **Wright, A. D. G., Kennedy, P., O'Neill,C. J., Toovey, A. F., Popovski, S., Reaa, S. M., Pimma, C. L. and Klein, L. 2004**. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine. **22:** 3976–3985.

112. **Yanagita, K., Kamagata, Y., Kawaharasaki, M., Suzuki, T., Nakamura, Y. and Minato, H. 2000.** Phylogenetic Analysis of Methanogens in Sheep Rumen Ecosystem and Detection of *Methanomicrobium mobile* by Fluorescence *in situ* Hybridization. Biosci. Biotechnol. Biochem. **64:** 1737-1742.

113. **Yang, C. –M. J. and Russell, J. B. 1993.** Effect of Monensin on the Specific Activity of Ammonia Production by Ruminal Bacteria and Disappearance of Ammonia Nitrogen from the Rumen. Appl. Environ. Microbiol. **59:** 3250-3254.

114. **Zinder, S. H. 1998.** Methanogens. Pages 113-132 in R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, G. Sayler, ed. Techniques in Microbial Ecology, Oxford University Press, USA.

8.0 APPENDIX

Table 8.1: Percentage of Total Bacteria Over time With or Without Monensin on Forage Diet

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
Phylum Verrucomicrobia	3.52	3.43	4.57	4.62	3.23	3.45	3.46	4.67	4.57	3.60	3.29	4.36
C[a]. Verrucomicrobiae	100	100	100	100	100	100	100	100	100	100	100	100
O[b]. Verrucomicrobiales	100	100	100	100	100	100	100	100	100	100	100	100
F[c].Verrucomicrobiaceae	100	100	100	100	100	100	100	100	100	100	100	100
Phylum Deinococcus Thermus	0.009	0.009	0.011	0.012	0.008	0.009	0.009	0.012	0.011	0.009	0.008	0.011
C. Deinococci	100	100	100	100	100	100	100	100	100	100	100	100
O. Deinococcales	100	100	100	100	100	100	100	100	100	100	100	100
F. Trueperaceae	100	100	100	100	100	100	100	100	100	100	100	100
Phylum Lentisphaerae	0.018	0.017	0.023	0.023	0.016	0.017	0.017	0.023	0.023	0.018	0.016	0.022
C. Lentisphaerae	100	100	100	100	100	100	100	100	100	100	100	100
O. Victivallales	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
F. Victivallaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC[d]. Lentisphaerae	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
Phylum Fusobacteria	0.018	0.017	0.057	0.058	0.040	0.017	0.017	0.047	0.057	0.045	0.041	0.055
C. Fusobacteria	100	100	100	100	100	100	100	100	100	100	100	100
O. Fusobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
F. Fusobacteriaceae	50.00	50.00	80.00	80.00	80.00	50.00	50.00	100	80.00	80.00	80.00	80.00
F. Incertae sedis 11	50.00	50.00	20.00	20.00	20.00	50.00	50.00	0.00	20.00	20.00	20.00	20.00
Phylum Deferribacteres	0.00	0.00	0.00	0.00	0.008	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C. Deferribacteres	0.00	0.00	0.00	0.00	100	0.00	0.00	0.00	0.00	0.00	0.00	0.00
O. Deferribacterales	0.00	0.00	0.00	0.00	100	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F. Incertae Sedis 3	0.00	0.00	0.00	0.00	100	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phylum Bacteroidetes	0.78	0.33	0.44	1.13	0.79	0.34	0.34	0.46	0.45	0.32	0.80	1.06
C. Bacteroidetes	68.54	15.38	13.16	65.98	65.31	15.38	15.38	15.38	15.38	13.89	65.98	65.98
O. Bacteroidales	100	100	100	100	100	100	100	100	100	100	100	100
F. Rikenellaceae	1.64	16.67	0.00	1.56	1.56	16.67	16.67	16.67	16.67	16.67	1.56	1.56

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Bacteroidaceae	96.72	66.67	80.00	96.88	96.88	66.67	66.67	66.67	66.67	66.67	96.88	96.88
UC. Bacteroidales	1.64	16.67	20.00	1.56	1.56	16.67	16.67	16.67	16.67	16.67	1.56	1.56
C. Sphingobacteria	5.62	15.38	15.79	6.19	7.14	15.38	15.38	15.38	15.38	17.14	6.19	6.19
O. Sphingobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
Family Flexibacteraceae	0.00	16.67	16.67	16.67	28.57	16.67	16.67	16.67	16.67	16.67	16.67	16.67
F. Sphingobacteriaceae	60.00	50.00	50.00	50.00	42.86	50.00	50.00	50.00	50.00	50.00	50.00	50.00
F. Saprospiraceae	20.00	16.67	16.67	16.67	14.29	16.67	16.67	16.67	16.67	16.67	16.67	16.67
F. Crenotrichaceae	20.00	16.67	16.67	16.67	14.29	16.67	16.67	16.67	16.67	16.67	16.67	16.67
C. Flavobacteria	7.87	28.21	28.95	11.34	11.22	28.21	28.21	28.21	28.21	20.00	11.34	11.34
O. Flavobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
F. Flavobacteriaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC. Bacteroidetes	17.98	41.03	42.11	16.49	16.33	41.03	41.03	41.03	41.03	45.71	16.49	16.49
Phylum Spirochaetes	0.13	0.13	0.011	0.012	0.13	0.13	0.13	0.012	0.011	0.14	0.12	0.00
C. Spirochaetes	100	100	100	100	100	100	100	100	100	100	100	0.00
O. Spirochaetales	100	100	100	100	100	100	100	100	100	100	100	0.00
F. Leptospiraceae	15.38	15.38	0.00	0.00	12.5	15.38	15.38	0.00	0.00	15.38	15.38	0.00
F. Spirochaetaceae	86.67	86.67	100	100	87.5	86.67	86.67	100	100	86.67	86.67	0.00
Phylum Actinobacteria	3.05	3.15	1.20	2.00	2.72	3.17	2.91	1.73	1.67	3.33	3.07	1.60
C. Actinobacteria	100	100	100	100	100	100	100	100	100	100	100	100
SubC[e]. Coriobacteridae	80.92	79.56	60.95	37.21	86.39	79.35	86.90	42.86	43.84	78.65	77.87	41.10
O. Coriobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
SubC. Actinobacteridae	19.08	20.44	39.05	62.79	13.61	19.10	13.10	57.14	56.16	21.35	22.13	58.90
O. Bifidobacteriales	54.55	49.33	34.15	34.30	36.96	48.68	38.64	42.86	45.12	46.84	44.58	43.02
O. Actionmycetales	45.45	50.67	65.85	65.74	63.04	51.32	61.36	57.14	54.88	53.16	55.42	56.98
Phylum Tenericutes	0.035	0.10	0.15	0.070	0.11	0.10	0.080	0.082	0.15	0.099	0.11	0.14
C. Mollicutes	100	100	100	100	100	100	100	100	100	100	100	100
O. Anaeroplasmatales	0.00	25.00	23.08	16.67	23.08	25.00	33.33	42.86	23.08	9.09	23.08	23.08
F. Anaeroplasmataceae	0.00	100	100	100	100	100	100	100	100	100	100	100

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
O. Acholeplasmatales	0.00	8.33	7.69	16.67	7.69	8.33	11.11	14.29	7.69	9.09	7.69	7.69
F. Acholeplasmataceae	0.00	100	100	100	100	100	100	100	100	100	100	100
O. Mycoplasmatales	100	66.67	69.23	66.67	69.23	66.67	55.56	42.86	69.23	81.82	69.23	69.23
F. Mycoplasmataceae	100	100	100	100	100	100	100	100	100	100	100	100
Phylum Firmicutes	87.06	87.68	87.78	84.75	88.01	87.34	88.13	86.77	85.57	87.92	87.17	85.51
C. Bacilli	3.05	3.19	3.79	3.37	3.22	2.35	2.43	1.81	1.97	0.89	3.40	2.97
O. Bacillales	26.49	25.15	28.18	23.17	23.30	12.61	22.67	59.70	55.78	79.31	22.65	35.34
F. Paenibacillaceae	2.50	4.88	4.88	7.02	4.88	13.33	7.14	2.50	4.88	5.80	4.88	4.88
F. Incertae Sedis11	10.00	9.75	9.75	14.04	9.75	26.67	14.29	10.00	9.75	0.00	9.75	9.75
F. Bacillaceae	35.00	34.15	34.15	49.12	34.15	13.33	50.00	35.00	34.15	39.13	34.15	34.15
F. Planococcaceae	8.75	8.54	8.54	15.79	8.54	20.00	12.50	8.75	8.54	8.70	8.54	8.54
F. Staphylococcaceae	43.75	42.68	42.68	14.04	42.68	26.67	16.07	43.75	42.68	46.38	42.68	42.68
O. Lactobacillales	73.51	74.85	71.82	76.83	76.70	87.39	77.33	40.30	44.22	20.69	77.35	64.66
F. Leuconostocaceae	0.45	0.41	0.48	1.06	0.37	0.48	0.52	0.00	1.54	0.00	0.35	2.67
F. Aerococcaceae	1.80	1.64	1.91	1.59	1.48	1.44	1.57	5.56	6.15	16.67	1.43	2.67
F. Lactobacillaceae	24.32	29.10	17.22	4.23	30.00	19.71	13.61	42.59	55.38	33.33	32.14	56.67
F. Enterrococcaceae	0.90	3.28	3.83	1.06	2.96	3.85	1.05	14.81	3.08	11.11	2.86	5.33
F. Streptococcaceae	70.72	63.93	75.60	89.95	63.70	73.56	82.20	35.19	30.77	33.33	61.79	30.00
F. Carnobacteriaceae	1.80	1.64	0.96	2.12	1.48	0.96	1.05	1.85	3.08	5.56	1.43	2.67
C. Erysipelotrichi	0.51	1.28	1.73	1.79	3.12	1.29	1.28	1.41	1.73	1.09	1.26	1.71
O. Erysipelotrichales	100	100	100	100	100	100	100	100	100	100	100	100
F. Erysipelotrichaceae	100	100	100	100	100	100	100	100	100	100	100	100
C. Clostridia	96.36	95.45	94.38	94.74	93.58	96.28	96.22	96.69	96.21	97.93	95.26	95.23
O. Clostridiales	98.44	98.76	97.68	97.80	98.57	98.45	98.50	97.97	97.97	98.47	98.23	97.83
F. Incertae Sedis13	0.30	0.30	0.35	0.41	0.28	0.16	0.26	0.09	0.09	0.06	0.28	0.38
F. Incertae Sedis11	0.04	0.04	0.00	0.00	0.04	0.04	0.04	0.00	0.00	0.04	0.04	0.00
F.Peptostreptococcaceae	0.14	0.13	0.20	0.19	0.17	0.15	0.15	0.19	0.21	0.14	0.14	0.19
F. Incertae Sedis 15	0.06	0.06	0.08	0.09	0.07	0.06	0.06	0.00	0.10	0.05	0.06	0.08

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Peptococcaceae	0.04	0.04	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.05	0.05
F. Eubacteriaceae	0.03	0.03	0.04	0.04	0.03	0.03	0.03	0.04	0.04	0.03	0.03	0.04
F. Veillonellaceae	1.94	1.88	0.86	3.89	2.71	0.69	0.71	0.94	0.92	0.56	2.76	3.77
F. Clostridiaceae	0.04	0.94	2.14	2.19	0.91	1.55	0.94	2.13	2.13	0.95	0.91	2.07
F. Ruminococcaceae	38.37	36.74	14.87	15.07	37.12	36.89	37.36	14.58	14.63	36.53	36.88	14.27
F. Lachnospiraceae	57.87	58.99	80.66	77.63	57.54	59.55	59.32	81.24	81.05	60.79	57.67	78.30
UC. Clostridiales	1.16	0.88	0.79	0.49	1.10	0.89	1.13	0.80	0.82	0.84	1.17	0.84
UC. Clostridia	1.56	1.24	2.32	2.20	1.43	1.55	1.50	2.03	2.03	1.53	1.77	2.16
UC. Firmicutes	0.08	0.08	0.09	0.10	0.07	0.08	0.08	0.09	0.09	0.08	0.08	0.09
Phylum Proteobacteria	4.94	4.71	5.14	6.68	4.48	4.98	4.44	5.56	6.87	4.04	4.92	6.65
C. Alphaproteobacteria	6.24	6.36	11.58	9.04	6.29	8.82	10.14	14.14	11.02	11.58	10.33	12.50
O. Caulobacterales	2.86	2.86	1.92	1.92	2.86	0.00	1.92	13.43	12.12	1.92	14.52	11.84
F. Caulobacteraceae	100	100	100	100	100	0.00	100	100	100	100	100	100
O. Rickettsiales	5.71	5.71	3.85	3.85	5.71	3.92	3.85	13.43	13.64	3.85	3.23	11.84
F. Anaplasmataceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Sphingomonadales	11.43	11.43	7.69	7.69	11.43	7.84	7.69	5.97	6.06	7.69	6.45	5.26
F. Sphingomonadaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Rhodobacterales	5.71	5.71	7.69	7.69	5.71	7.84	7.69	5.97	6.06	7.69	6.45	15.79
F. Rhodobacteraceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Rhodospirillales	2.86	2.86	1.92	1.92	2.86	1.96	1.92	1.49	1.52	1.92	1.61	1.32
F. Acetobacteraceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Rhizobiales	60.00	60.00	69.23	69.23	60.00	70.59	69.23	53.73	54.55	69.23	58.06	48.68
F. Brucellaceae	33.33	33.33	19.44	19.44	33.33	19.44	19.44	19.44	19.44	19.44	19.44	18.92
F. Rhizobiaceae	23.81	23.81	27.78	27.78	23.81	27.78	27.78	27.78	27.78	27.78	27.78	27.03
F. Bartonellaceae	19.05	19.05	11.11	11.11	19.05	11.11	11.11	11.11	11.11	11.11	11.11	10.81
F. Bradyrhizobiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.22
F. Hyphomicrobiaceae	14.29	14.29	8.33	8.33	14.29	8.33	8.33	8.33	8.33	8.33	8.33	8.11
F. Phyllobacteriaceae	9.52	9.52	5.56	5.56	9.52	5.56	5.56	5.56	5.56	5.56	5.56	5.41

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Methylobacteriaceae	0.00	0.00	11.11	11.11	0.00	11.11	11.11	11.11	11.11	11.11	11.11	13.51
UC. Alphaproteobacteria	11.43	11.43	7.69	7.69	11.43	7.84	7.69	5.97	6.06	7.69	9.68	5.26
C. Deltaproteobacteria	8.91	9.09	10.91	8.87	8.99	8.65	8.55	10.55	8.35	11.14	8.50	8.39
O. Syntrophobacterales	2.00	2.00	2.04	1.96	2.00	2.00	2.04	2.00	2.00	2.00	1.96	1.96
F. Syntrophaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Desulfobacterales	2.00	2.00	2.04	1.96	2.00	2.00	2.04	2.00	2.00	2.00	1.96	1.96
F. Desulfobaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Desulfovibrionales	94.00	94.00	93.88	94.12	94.00	94.00	93.88	94.00	94.00	94.00	94.12	94.12
F. Desulfovibrionaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC. Deltaproteobacteria	2.00	2.00	2.04	1.96	2.00	2.00	2.04	2.00	2.00	2.00	1.96	1.96
C. Epsilonproteobacteria	5.88	6.00	7.35	5.74	6.12	5.71	6.43	6.96	5.51	7.35	5.50	5.43
O. Campylobacterales	100	100	100	100	100	100	100	100	100	100	100	100
F. Campylobacteraceae	42.42	42.42	42.42	42.42	41.18	42.42	42.42	42.42	42.42	42.42	42.42	42.42
F. Helicobacteraceae	57.58	57.58	57.58	57.58	58.82	57.58	57.58	57.58	57.58	57.58	57.58	57.58
C. Gammaproteobacteria	37.79	37.27	20.04	36.00	36.51	37.02	30.02	32.91	36.56	31.85	36.50	35.53
O. Chromatiales	0.47	0.49	1.11	0.48	0.49	0.47	0.65	0.00	0.46	0.00	0.46	0.46
F. Chromatiaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Pasteurellales	6.60	0.98	15.56	6.76	12.81	4.21	9.09	8.97	6.39	1.40	6.39	6.48
F. Pasteurellaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Xanthomonadales	12.26	12.68	26.67	10.63	13.30	12.32	3.90	3.85	12.33	4.20	12.33	12.50
F. Xanthomonadaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Vibrionales	0.47	0.49	1.11	0.48	0.49	0.47	0.65	0.00	0.46	0.64	0.46	0.46
F. Vibrionaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Pseudomonadales	4.24	5.85	13.33	4.35	7.88	5.61	7.79	1.92	5.48	2.10	5.48	4.17
F. Moraxellaceae	100	91.67	91.67	100	93.75	91.67	91.67	66.67	91.67	66.67	91.67	100
UC. Pseudomonadales	0.00	8.33	8.33	0.00	6.25	8.33	8.33	33.33	8.33	33.33	8.33	0.00
O. Aeromonadales	1.42	1.95	5.56	0.48	2.46	2.34	0.65	1.28	2.28	0.70	2.28	2.31
F. Aeromonadaceae	100	75.00	60.00	0.00	60.00	60.00	0.00	0.00	60.00	0.00	60.00	60.00

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Succinivibrionaceae	0.00	25.00	40.00	100	40.00	40.00	100	100	40.00	100	40.00	40.00
O. Enterobacteriales	66.98	69.76	18.89	69.08	54.68	66.82	66.88	83.33	65.30	90.90	65.30	66.2
F. Enterobacteriaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC. Gammaproteobacteria	7.55	7.80	17.78	7.73	7.88	7.48	10.39	0.00	7.31	0.00	7.31	7.41
C. Betaproteobacteria	40.82	40.91	49.67	40.00	41.73	39.45	43.47	35.02	38.23	37.64	38.83	37.83
O. Rhodocyclales	0.00	0.44	0.45	0.43	0.43	0.44	0.45	0.60	0.44	0.60	0.43	0.43
F. Rhodocyclaceae	0.00	100	100	100	100	100	100	100	100	100	100	100
O. Neisseriales	5.68	5.78	3.14	5.65	5.60	5.70	3.14	3.61	5.68	3.55	5.58	5.65
F. Neisseriaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Burkholderiales	93.89	93.33	95.96	93.04	93.10	93.42	95.96	95.78	93.45	95.86	93.13	93.04
F. Comamonadaceae	14.88	12.86	14.95	14.95	14.81	14.08	14.95	18.87	14.95	18.52	14.75	14.95
F. Burkholderiaceae	2.79	2.86	2.80	2.80	2.78	2.82	2.80	1.89	2.80	1.85	2.76	2.80
F. Oxalobacteraceae	13.95	14.29	13.55	14.02	13.43	14.08	13.55	18.24	14.02	18.52	13.82	14.02
F. Alcaligenaceae	59.07	60.48	58.88	58.41	58.80	59.62	59.35	47.80	58.41	47.53	58.53	58.41
F. Incertae Sedis 5	0.00	0.00	0.93	0.93	0.93	0.00	0.00	0.00	0.93	1.23	0.92	0.93
UC. Burkholderiales	9.30	9.52	8.88	8.88	9.26	9.39	9.35	11.95	8.88	12.35	9.22	8.88
UC. Betaproteobacteria	0.44	0.44	0.45	0.87	0.86	0.44	0.45	0.00	0.44	0.00	0.86	0.87
UC. Proteobacteria	0.36	0.36	0.45	0.35	0.36	0.35	0.39	0.42	0.33	0.45	0.33	0.33
UC. Bacteria	0.44	0.42	0.62	0.65	0.44	0.45	0.48	0.63	0.62	0.49	0.46	0.60

[a]: Class; [b]: Order; [c]: Family; [d]: Unclassified; [e]: Subclass

+ : Monensin supplemented; - : Control

Table 8.2: Percentage of Bacteria over time with/without Monensin on Concentrate Diet

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
Phylum Verrucomicrobia	3.26	3.55	3.66	3.09	3.41	3.42	3.49	3.25	4.53	3.37	4.49	4.60
C[a]. Verrucomicrobiae	100	100	100	100	100	100	100	100	100	100	100	100
O[b]. Verrucomicrobiales	100	100	100	100	100	100	100	100	100	100	100	100
F[c].Verrucomicrobiaceae	100	100	100	100	100	100	100	100	100	100	100	100
Phylum Deinococcus Thermus	0.008	0.009	0.009	0.008	0.009	0.009	0	0.008	0.011	0.008	0.011	0.012
C. Deinococci	100	100	100	100	100	100	100	100	100	100	100	100
O. Deinococcales	100	100	100	100	100	100	100	100	100	100	100	100
F. Trueperaceae	100	100	100	100	100	100	100	100	100	100	100	100
Phylum Lentisphaerae	0.016	0.018	0.018	0.016	0.017	0.017	0.017	0.016	0.023	0.017	0.023	0.023
C. Lentisphaerae	100	100	100	100	100	100	100	100	100	100	100	100
O. Victivallales	50	50	50	50	50	50	50	50	50	50	50	50
F. Victivallaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC[d]. Lentisphaerae	50	50	50	50	50	50	50	50	50	50	50	50
Phylum Fusobacteria	0.041	0.044	0.046	0.031	0.043	0.043	0.044	0.041	0.057	0.042	0.056	0.058
C. Fusobacteria	100	100	100	100	100	100	100	100	100	100	100	100
O. Fusobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
F. Fusobacteriaceae	80	80	80	75	80	80	80	80	80	80	80	80
F. Incertae sedis 11	20	20	20	25	20	20	20	20	20	20	20	20
Phylum Deferribacteres	0	0	0	0	0	0	0	0	0	0	0.011	0
C. Deferribacteres	0	0	0	0	0	0	0	0	0	0	100	0
O. Deferribacterales	0	0	0	0	0	0	0	0	0	0	100	0
F. Incertae Sedis 3	0	0	0	0	0	0	0	0	0	0	100	0
Phylum Bacteroidetes	0.79	0.34	0.31	0.30	0.31	0.34	0.34	0.79	1.06	0.33	1.06	1.08
C. Bacteroidetes	65.98	16	15	13	19	18	15	66	69	15	65	65
O. Bacteroidales	100	100	100	100	100	100	100	100	100	100	100	100
F. Rikenellaceae	1.56	17	0	0	14	14	17	1.56	1.56	17	1.64	1.64
F. Bacteroidaceae	96.88	66	80	80	72	72	66	96.88	96.88	66	96.72	96.72
UC. Bacteroidales	1.56	17	20	20	14	14	17	1.56	1.56	17	1.64	1.64

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
C. Sphingobacteria	6.19	13.16	17.64	25.64	16.67	15	15.38	6.19	6.45	15.38	6.38	6.38
O. Sphingobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
F. Flexibacteraceae	16.67	0	16.67	40	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67
F. Sphingobacteriaceae	50	60	50	30	50	50	50	50	50	50	50	50
F. Saprospiraceae	16.67	20	16.67	10	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67
F. Crenotrichaceae	16.67	20	16.67	10	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67
UC. Sphingobacteriales	0	0	0	10	0	0	0	0	0	0	0	0
C. Flavobacteria	11.34	28.95	20.59	20.51	19.44	46.94	28.21	11.34	7.53	28.21	11.70	11.70
O. Flavobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
F. Flavobacteriaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC. Bacteroidetes	16.49	42.10	47.06	41.03	44.44	40	41.03	16.49	17.20	41.03	17.02	17.02
Phylum Spirochaetes	0.12	0.13	0.14	0.12	0.13	0.13	0.13	0.12	0.011	0.13	0.011	0.012
C. Spirochaetes	100	100	100	100	100	100	100	100	100	100	100	100
O. Spirochaetales	100	100	100	100	100	100	100	100	100	100	100	100
F. Leptospiraceae	13.33	13.33	13.33	12.5	13.33	13.33	13.33	13.33	0	13.33	0	0
F. Spirochaetaceae	86.67	86.67	86.67	87.5	86.67	86.67	86.67	86.67	100	86.67	100	100
Phylum Actinobacteria	3.17	3.25	3.01	2.44	3.31	3.19	3.22	3.14	1.19	3.07	1.68	1.52
C. Actinobacteria	100	100	100	100	100	100	100	100	100	100	100	100
SubC[e]. Coriobacteridae	74.68	79.56	87.84	86.98	75.06	78.07	78.32	75.45	51.43	80	41.61	46.21
O. Coriobacteriales	100	100	100	100	100	100	100	100	100	100	100	100
SubC. Actinobacteridae	25.32	20.44	12.16	13.02	24.94	21.93	21.68	24.55	48.57	20	58.39	53.79
O. Bifidobacteriales	37.37	49.33	35	31.71	38.14	45.12	45	38.95	33.33	49.32	42.53	52.11
O. Actionmycetales	62.63	50.67	65	68.29	61.86	54.88	55	61.05	66.67	50.68	57.47	47.89
Phylum Tenericutes	0.073	0.062	0.055	0.078	0.077	0.11	0.11	0.11	0.14	0.08	0.15	0.14
C. Mollicutes	100	100	100	100	100	100	100	100	100	100	100	100
O. Anaeroplasmatales	33.33	42.86	0	0	0	23.08	25	23.08	25	33.33	23.08	25
F. Anaeroplasmataceae	100	100	0	0	0	100	100	100	100	100	100	100
O. Acholeplasmatales	11.11	0	16.67	0	11.11	7.69	8.33	7.69	8.33	11.11	7.69	8.33

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Acholeplasmataceae	100	0	100	0	100	100	100	100	100	100	100	100
O. Entomoplasmatales	0	0	0	10	0	0	0	0	0	0	0	0
F. Spiroplasmataceae	0	0	0	100	0	0	0	0	0	0	0	0
O. Mycoplasmatales	55.56	57.14	83.33	90	88.89	69.23	66.67	69.23	66.67	55.56	69.23	66.67
F. Mycoplasmataceae	100	100	100	100	100	100	100	100	100	100	100	100
Phylum Firmicutes	87.29	86.93	89.88	90.23	87.17	87.41	88.03	87.22	86.29	87.55	85.37	86.23
C. Bacilli	3.29	2.27	2.24	2.27	2.69	2.74	2.35	3.44	3.57	2.91	3.39	3.79
O. Bacillales	23.16	10.31	25	30.30	29.82	29.18	22.36	22.70	21.03	27.06	28.19	28.98
F .Paenibacillaceae	4.88	8.70	7.27	5	4.88	4.88	7.55	4.76	7.02	4.88	4.76	4.88
F. Incertae Sedis 11	9.76	34.78	14.55	10	9.76	9.76	15.09	9.52	14.04	9.76	9.52	9.76
F. Bacillaceae	34.15	8.70	7.27	35	34.15	34.15	7.55	33.33	49.12	34.15	33.33	34.15
F. Planococcaceae	8.54	13.04	9.09	7.5	8.54	8.54	9.43	10.71	12.28	8.54	10.71	8.54
F. Staphylococcaceae	42.68	34.78	61.82	42.5	42.68	42.68	60.38	41.67	17.54	42.68	41.67	42.68
O. Lactobacillales	76.84	89.69	75	69.70	70.18	70.82	77.64	77.30	78.97	72.94	71.81	71.02
F. Leuconostocaceae	0.37	0.5	0	0.54	1.04	0.50	0	1.40	1.40	1.36	0.47	0
F. Aerococcaceae	1.47	1.5	1.82	1.63	2.07	2.01	1.63	1.40	1.87	1.81	1.87	1.49
F. Lactobacillaceae	30.15	17	3.03	10.87	9.84	13.07	11.96	32.52	11.68	20.36	19.16	15.92
F. Enterrococcaceae	2.94	3	1.21	1.09	4.15	4.02	1.09	2.80	3.74	3.62	3.74	3.98
F. Streptococcaceae	63.60	77	93.33	85.33	81.35	79.40	84.78	60.49	79.44	71.95	73.83	77.61
F. Carnobacteriaceae	1.47	1	0.61	0.54	1.55	1.01	0.54	1.40	1.87	0.90	0.93	0.50
C. Erysipelotrichi	1.22	1.19	0.46	1.60	0.57	1.29	1.11	1.24	1.75	1.26	1.73	1.75
O. Erysipelotrichales	100	100	100	100	100	100	100	100	100	100	100	100
F. Erysipelotrichaceae	100	100	100	100	100	100	100	100	100	100	100	100
C. Clostridia	95.41	96.45	97.29	96.06	96.67	95.90	96.46	95.25	94.59	95.75	94.25	94.37
O. Clostridiales	98.51	98.44	98.83	98.46	98.45	98.44	98.59	98.19	98.01	98.21	97.70	97.96
F. Incertae Sedis 13	0.28	0.16	0.16	0.45	0.29	0.29	0.26	0.28	0.40	0.26	0.40	0.41
F. Incertae Sedis 11	0.05	0.04	0.04	0.12	0.04	0.04	0.04	0.04	0	0.04	0	0
F. Peptostreptococacae	1.46	0.16	0.14	0.14	0.14	0.13	0.14	0.49	0.20	0.14	0.23	0.20

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Incertae Sedis 15	0.06	0.06	0.05	0.05	0.06	0.06	0.05	0.06	0.09	0.06	0.10	0.07
F. Eubacteriaceae	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.043	0.34	0.04	0.04
F. Veillonellaceae	2.76	0.61	0.68	0.68	0.75	0.72	0.68	2.77	2.87	0.73	1.00	0.96
F. Clostridiaceae	1.47	0.03	0.93	1.11	1.55	0.94	0.92	1.50	1.29	1.54	1.30	1.32
F. Ruminococcaceae	35.88	36.46	36.38	35.24	37.13	37.38	37.27	36.37	13.82	37.45	14.70	14.07
F. Lachnospiraceae	56.81	61.55	60.53	61.08	58.89	59.24	59.80	57.04	80.84	58.62	81.39	82.50
F. Peptococcaceae	0	0	0	0	0	0	0	0.04	0	0	0	0
UC. Clostridiales	1.15	0.88	1.06	1.10	1.11	1.17	0.81	1.18	0.45	1.12	0.83	0.43
UC. Clostridia	1.49	1.56	1.17	1.54	1.55	1.56	1.41	1.72	1.99	1.79	2.30	2.04
UC. Firmicutes	0.07	0.08	0.01	0.07	0.08	0.08	0.08	0.07	0.09	0.08	0.09	0.09
Phylum Proteobacteria	4.78	5.20	2.47	3.25	5.07	4.87	4.17	4.86	6.15	4.94	6.53	5.76
C. Alphaproteobacteria	8.32	8.86	17.87	13.60	10.07	6.13	8.81	10.18	9.80	8.86	8.28	7.01
O. Caulobacterales	2.04	1.92	2.08	0	1.67	2.86	2.38	1.64	1.89	1.92	2.08	2.86
F. Caulobacteraceae	100	100	100	0	100	100	100	100	100	100	100	100
O. Rickettsiales	4.08	3.85	4.17	15.79	3.33	5.71	21.43	4.92	3.77	3.85	4.17	5.71
F. Anaplasmataceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Sphingomonadales	8.16	7.69	8.33	7.02	6.67	11.43	9.52	6.56	7.55	7.69	8.33	11.43
F. Sphingomonadaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Rhodobacterales	8.16	7.69	8.33	7.02	20	5.71	4.76	19.67	7.55	7.69	8.33	5.71
F. Rhodobacteraceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Rhodospirillales	2.04	1.92	2.08	0	1.67	2.86	2.38	1.64	1.89	1.92	2.08	2.86
F. Acetobacteraceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Rhizobiales	67.35	69.23	66.67	63.16	60	60	50	59.02	69.81	69.23	66.67	60
F. Brucellaceae	21.21	19.44	21.88	19.44	19.44	33.33	33.33	19.44	18.92	19.44	21.88	33.33
F. Rhizobiaceae	18.18	27.78	18.75	27.78	27.78	23.81	23.81	27.78	27.03	27.78	18.75	23.81
F. Bartonellaceae	12.12	11.11	12.5	11.11	11.11	19.05	19.05	11.11	10.81	11.11	12.5	19.05
F. Bradyrhizobiaceae	18.18	16.67	18.75	16.67	16.67	0	0	16.67	16.22	16.67	18.75	0
F. Hyphomicrobiaceae	9.09	8.33	9.38	8.33	8.33	14.29	14.29	8.33	8.11	8.33	9.38	14.29

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Phyllobacteriaceae	6.06	5.56	6.25	5.56	5.56	9.52	9.52	5.56	5.41	5.56	6.25	9.52
F.Methylobacteriaceae	15.15	11.11	12.5	11.11	11.11	0	0	11.11	13.51	11.11	12.5	0
UC. Alphaproteobacteria	8.16	7.69	8.33	7.02	6.67	11.43	9.52	6.56	7.55	7.69	8.33	11.43
C. Deltaproteobacteria	8.66	8.52	18.15	11.69	8.39	8.76	10.48	8.51	9.24	8.52	8.62	9.82
O. Syntrophobacterales	1.96	2.00	2.04	2.04	2.00	2.00	2.00	1.96	2.00	2.00	2.00	0
F. Syntrophaceae	100	100	100	100	100	100	100	100	100	100	100	0
O. Desulfobacterales	1.96	2.00	2.04	2.04	2.00	2.00	2.00	1.96	2.00	2.00	2.00	2.04
F. Desulfobaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Desulfovibrionales	94.12	94.00	93.88	93.88	94.00	94.00	94.00	94.12	94.00	94.00	94.00	94.74
F. Desulfovibrionaceae	100	100	100	100	100	100	100	100	100	100	100	100
UC. Deltaproteobacteria	1.96	2.00	2.04	2.04	2.00	2.00	2.00	1.96	2.00	2.00	2.00	2.04
C. Epsilonproteobacteria	5.60	5.62	12.22	7.88	5.54	5.78	6.92	5.51	6.10	5.62	5.86	6.61
O. Campylobacterales	100	100	100	100	100	100	100	100	100	100	100	100
F.Campylobacteraceae	42.42	42.42	42.42	42.42	42.42	42.42	42.42	42.42	42.42	42.42	41.18	42.42
F. Helicobacteraceae	57.58	57.58	57.58	57.58	57.58	57.58	57.58	57.58	57.58	57.58	28.82	57.58
C. Gammaproteobacteria	37.35	37.31	10.74	17.90	36.58	38.35	37.32	36.56	32.16	37.31	37.24	38.08
O. Chromatiales	0.45	0.46	3.45	1.33	0.46	0.46	0.56	0.46	0.57	0.46	0.046	0
F. Chromatiaceae	100	100	100	100	100	100	100	100	100	100	100	0
O. Pasteurellales	6.36	6.39	6.90	37.33	6.42	6.39	7.87	6.39	1.15	6.39	6.48	4.83
F. Pasteurellaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Xanthomonadales	12.27	12.33	10.34	4.00	12.39	12.33	3.37	12.33	15.52	12.33	12.50	13.68
F. Xanthomonadaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Vibrionales	0.45	0.46	0	1.33	0.46	0.46	0.56	0.46	0.57	0.46	0.046	0.53
F. Vibrionaceae	100	100	0	100	100	100	100	100	100	100	100	100
O. Pseudomonadales	5.45	5.48	17.24	22.67	5.50	5.48	4.49	5.48	6.90	5.48	4.17	0.53
F. Moraxellaceae	91.67	91.67	100	100	91.67	91.67	87.5	91.67	91.67	91.67	100	100
UC. Pseudomonadales	8.33	8.33	0	0	8.33	8.33	12.5	8.33	8.33	8.33	0	0
O. Aeromonadales	2.27	2.28	3.45	2.67	1.83	2.28	1.12	2.28	2.30	2.28	2.31	2.63

Microbial Level	Week 1		Week 3		Week 4		Week 6		Week 10		Week 12	
	+	-	+	-	+	-	+	-	+	-	+	-
F. Aeromonadaceae	60.00	60.00	0	0	75.00	60.00	0	60.00	75.00	60.00	60.00	60.00
F. Succinivibrionaceae	40.00	40.00	100	100	25.00	40.00	100	40.00	25.00	40.00	40.00	40.00
O. Enterobacteriales	65.00	65.30	3.45	6.67	65.60	65.30	73.03	65.30	63.79	65.30	66.20	75.26
F. Enterobacteriaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Altermonadales	0	0	0	2.67	0	0	0	0	0	0	0	0
FPseudoaltermonadcae	0	0	0	100	0	0	0	0	0	0	0	0
UCGammaproteobacteria	7.27	7.31	55.17	21.33	7.34	7.31	8.99	7.31	9.20	7.31	7.41	0
C. Betaproteobacteria	39.73	39.35	40.74	48.45	39.09	40.63	36.06	38.90	42.33	39.35	39.66	38.08
O. Rhodocyclales	0.43	0.43	0.91	0.49	0.43	0.43	0.58	0.43	0.44	0.43	0.43	0.53
F. Rhodocyclaceae	100	100	100	100	100	100	100	100	100	100	100	100
O. Neisseriales	5.56	5.63	0	0.49	5.58	5.60	3.49	5.58	5.68	5.63	5.65	6.32
F. Neisseriaceae	100	100	0	100	100	100	100	100	100	100	100	100
O. Burkholderiales	93.16	93.51	98.18	98.52	93.56	93.53	95.35	93.13	93.01	93.51	93.48	93.16
F. Comamonadaceae	14.68	13.89	8.33	15.00	14.68	14.75	19.51	14.75	14.08	14.81	14.88	16.95
F. Burkholderiaceae	2.75	2.78	0	3.00	2.75	2.76	1.83	2.76	2.82	2.78	2.79	1.69
F. Oxalobacteraceae	13.76	13.89	0.93	14.5	13.76	13.82	17.68	13.82	13.62	13.43	13.95	16.95
F. Incertae Sedis 5	0.92	0.93	1.85	1.00	0.92	0.92	1.22	0.92	0.94	0.93	0.93	1.13
F. Alcaligenaceae	58.72	59.26	70.37	6.00	58.72	58.53	47.56	58.53	59.62	58.80	58.60	52.54
UC. Burkholderiales	9.17	9.26	18.52	10.5	9.17	9.22	12.20	9.22	8.92	9.26	8.84	10.73
UC. Betaproteobacteria	0.85	0.43	0.91	0.49	0.43	0.43	0.58	0.86	0.87	0.43	0.43	0
UC. Proteobacteria	0.34	0.34	0.37	0.48	0.34	0.35	0.42	0.33	0.37	0.34	0.34	0.40
UC. Bacteria	0.45	0.46	0.40	0.42	0.45	0.47	0.45	0.45	0.55	0.47	0.61	0.55

[a]: Class; [b]: Order; [c]: Family; [d]: Unclassified; [e]: Subclass

+ : Monensin supplemented; - : Control