Methane emissions and rumen microbial changes in steers fed condensed tannin containing diets under western Canadian conditions.

by

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

MASTER OF SCIENCE

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ABSTRACT

A study was conducted to determine if sainfoin, a condensed tannin (CT) containing legume, fed to beef cattle as hay or silage during a western Canadian winter would result in a reduction in methane (CH_4) emissions without compromising animal performance.

Forty yearling beef steers were fed four diets in a factorial design consisting of two legume forage species (sainfoin or alfalfa) and two preservation methods (silage or hay) over 15 weeks (wks). For each sample wk, animal weight, 24-h CH_4 expiration and rumen fluid samples were obtained. Rumen methanogens were characterized using terminal restriction fragment length polymorphisms analysis. Specific bacteria were quantified with real-time polymerase chain reaction analysis.

Sainfoin silage (SS) and sainfoin hay (SH) contained 11.9 and 10.5 mg g^{-1} of CT respectively and supported an acceptable growth rate for backgrounding steers. A decline ($P < 0.05$) in enteric CH_4 formation could only be detected from SH-fed animals compared to alfalfa hay (AH) fed animals when CH_4 was expressed as L d^{-1} or L kg BW^{-1}. The rumen archaeal community structure of experimental animals remained stable regardless of diet type or sample wk. Structural carbohydrate-fermenting bacteria were suppressed in silage diets. Methanogens were less abundant in the rumen fluid samples of steers fed SS but not SH.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Kim Ominski for her guidance and support throughout my undergraduate and graduate degree programs. You are truly an exceptional woman and I have learnt so much from you. Thank you to Dr. Krause for providing direction and advice throughout my graduate degree program. You introduced me to agriculture's microbial world and for that, believe it or not, I am grateful. Thanks are also extended to Dr. Wittenberg, Dr. McAllister and Dr. Holley for taking the time to provide assistance and act as committee members for this research project.

A huge thank you is extended to Terri Garner, Chad Malfait, Darcy Catellier and Glenlea's staff. Forty steers could not have been better cared for by anyone else. Your presence in this project made working in very cold weather a lot of fun. Thank you to Clayton Robins and the Brandon Research Center staff for their efforts and for providing feed for our animals.

Technicians Colleen Wilson, Janice Haines and Karen Lim are greatly appreciated for their assistance both in the lab and out at the barns. I could not have accomplished this project without you all. Thank you to both Ainsley Little and Shucong Lee for their invaluable guidance and support regarding the microbial aspect of this research. I would also like to extend my sincere gratitude toward Dr. Onischuk for her limitless patience and statistical guidance.

A very special thank you is expressed toward my two "partners in crime" Jenelle Bouchard and Natalie Berard. It was an absolute pleasure working with you both. Your endless support, reliability and friendship will never be forgotten.

Thank you to my fellow grad-students and the Animal Science Department in its entirety for making my graduate degree program a positive experience. I consider myself very fortunate to have had the opportunity to meet so many wonderful people.

I would like to extend love and gratitude to my family for their guidance, support and for instilling in me the importance of education. I would also like to thank my husband Kevin for his patience and encouragement throughout this process. I love you and am finally finished.

DEDICATION

I dedicate this work to my parents. Dad, it is because of you that I am so passionate about agriculture and cattle. You have taught me things that you cannot learn in a classroom. Mom, you have instilled in me the importance and value of education. From you both, I get my love and appreciation of animals….from whom else would I have learned how to hatch ducklings in an electric frying pan? Over the years you both have sacrificed so much for me and I am eternally grateful.

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 summary and conclusions. The format used to write this thesis is that of the Canadian Journal of Animal Science. The title of the manuscript is:

Methane emissions and rumen microbial changes in steers fed condensed tannin containing diets under western Canadian conditions.

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ABBREVIATIONS

ADG	average daily gain
ADF	acid detergent fiber
ATP	adenosine triphosphate
BW	body weight
CH_4	methane
CO_2	carbon dioxide
CoM	coenzyme M
CP	crude protein
CT	condensed tannins
DM	dry matter
DMI	dry matter intake
GEI	gross energy intake
Gt CO_2 –eq	gigatonnes of carbon dioxide equivalent
GWP	global warming potential
H_2	hydrogen
H_2O	water
H4MPT	tetrahydromethanopterin
HS-HTP	7-mercaptoheptanoylthreonine
MW	molecular weight
MFR	methanofuran
mV	megavolt
NAD	nicotinamide adenine dinucleotide (oxidized form)

NADH	nicotinamide adenine dinucleotide (reduced form)
NDF	neutral detergent fiber
PCR	polymerase chain reaction
ppb	parts per billion
ppm	parts per million
RFI	residual feed intake
RT-PCR	real time polymerase chain reaction
Tg	teragram
TRF	terminal restriction fragment
TRFLP	terminal restriction fragment length polymorphism
µm	micrometer
VFA	volatile fatty acid

1.0 GENERAL INTRODUCTION

In recent years, methane (CH_4) gas has gained increased recognition for its role in the greenhouse effect. Methane is second only to carbon dioxide (CO_2) in its contribution of estimated radiative forcing derived from anthropogenic sources (Lassey 2007) and has been designated a global warming potential (GWP) of 25 by the Intergovernmental Panel on Climate Change (IPCC 2007b). Ruminant livestock are identified as major contributors to global anthropogenic CH_4 production. Cattle typically lose from 3 to 12% of their ingested energy as eructated CH_4 (Johnson and Johnson 1995). These losses are not only an environmental concern but also create inefficiencies in cattle production. Reducing enteric CH_4 emissions from cattle would lessen the impact of livestock production on the globe as well as decrease costs of production by increasing feed efficiency. Considerable research has been conducted to decrease CH_4 formation from cattle including the selection of low CH_4-emitting cattle (Nkrumah et al. 2006), improving feed quality (Ominski et al. 2006), and inclusion of ionophores in the diet (Guan et al. 2006). For a strategy to be adopted on-farm, it must be easily executed, inexpensive and conform to consumer expectations.

Feeding condensed tannins (CT), a type of plant polyphenol, has lead to beneficial effects on ruminant animal performance including reduced enteric CH_4 production when incorporated in the diet at concentrations ranging between 2-4% of dry matter (DM) (Aerts et al. 1999). Within western Canada, plants that naturally contain CT such as *Onobrychis viciifolia* (sainfoin) and *Lotus corniculatus* (birdsfoot trefoil) are of interest to the beef cattle industry for their potential role as feedstuffs. Due to the multifaceted chemistry and fluctuating concentrations of CT within plants, further research is required

to determine if CT from legume forages grown in a Canadian production environment may improve cattle performance and lower CH_4 production.

In Canada, forages grown in the summer are preserved for winter feeding. It has been hypothesized that the extensive fermentation, which occurs during ensiling, decreases carbohydrate digestion in the rumen and consequently reduces CH_4 formation (Sundstøl, 1981). To date, little if any research has been conducted to examine the impact of forage preservation on enteric CH_4 formation.

To investigate the effects of CT on enteric CH_4 production it is critical to understand their effect on rumen bacterial ecology. Condensed tannins are generally regarded as inhibitory to the growth of rumen microorganisms because of their ability to complex with polymers (Jones et al. 1994), minerals (Scalbert 1991) and the bacterial cell surface (McSweeney et al. 2001). It is speculated that CT may affect rumen methanogenesis indirectly by reducing fiber digestion and ultimately decreasing hydrogen (H_2) production in the rumen or directly by inhibiting the growth of methanogens (Tavendale et al. 2005). The composition of rumen microbial populations can be assessed by employing molecular techniques such as terminal restriction fragment length polymorphisms (TRFLP) and real-time polymerase chain reaction (RT-PCR).

The purpose of this study was to determine the effect of feeding sainfoin grown in a Manitoba production environment on rumen fermentation, enteric CH_4 production and the rumen bacterial ecology of backgrounding steers during a Canadian winter. The effect of method of forage preservation (silage versus hay) on these parameters was also explored.

2.0 LITERATURE REVIEW

2.1 Methane and Climate Change

2.1.1 Methane

Methane (CH_4), a chemical compound with the molecular formula CH_4, is a colorless, odorless gas with a broad distribution in nature. Although CH_4 is well known as the principle component of natural gas, it has gained increased recognition for its role in the greenhouse effect (United States Environmental Protection Agency (U.S. EPA) 2006).

2.1.2 The Greenhouse Effect

The greenhouse effect is a natural process whereby energy from the sun passes through the earth's atmosphere, warming its surface. Approximately 31% of all incoming solar energy is directly reflected back into space by the earth's atmosphere and surfaces such as snow and ice (Environment Canada 2003). Twenty percent of all solar energy is absorbed by clouds, gases, such as ozone, and particles in the earth's atmosphere (Environment Canada 2003). The remaining solar energy, mainly in the form of visible light, is absorbed by the earth's oceans and land where it is converted into heat (Environment Canada 2003). This heat acts to warm the surface of the earth and the air above it. Some of this heat energy in the form of infra-red radiation (4-100 μm) is released back into the atmosphere. Seventy percent of the emitted radiation falls within the wavelength band of seven to thirteen μm and is able to pass through the atmosphere into space (Moss et al. 2000). The remaining radiation is absorbed by water vapor and greenhouse gases such as carbon dioxide (CO_2), CH_4 and nitrous oxide (N_2O). As the gas molecules warm they emit infrared radiation, some of which returns to the earth's surface,

resulting in further warming. In the absence of the natural greenhouse effect, life on this planet would probably not exist as the earth's average temperature would be -18˚C rather than the present day 15˚C (Environment Canada 2003). The amount of additional heat energy added to the earth's atmosphere is dependent upon the concentration of greenhouse gases. Since the beginning of the industrial revolution, the concentration of all greenhouse gases present in the atmosphere has increased (Pidwirny 2006). For example, pre-industrial global concentrations of CO_2 and CH_4 were 280 parts per million (ppm) and 715 parts per billion (ppb). By 2005, their concentrations had risen substantially to 379 ppm and 1774 ppb (Intergovernmental Panel on Climate Change (IPCC) 2007c). It is believed that higher greenhouse gas concentrations will enhance the natural greenhouse effect and as a result the earth's climate will become warmer. This process is more commonly known as "global warming".

2.1.3 Sources of Methane

Methane is a greenhouse gas with both natural and human-influenced sources. Johnson and Johnson (1995) reported that radiocarbon [^{14}C-] isotope measurements of atmospheric CH_4 indicate that between 20 and 30% of CH_4 is of fossil origin and the remaining 70 to 80% is derived from contemporary carbon. Sources contributing to mature carbon include gas drilling, venting and distribution, as well as mining and wetland emissions containing carbon that has been stored for thousands of years. Contemporary contributors of carbon include enteric fermentation from insects and ruminant animals, natural wetlands, biomass burning, oceans and lakes, rice production and waste treatment in landfills and sewers.

2.1.4 Methane's Role in Climate Change

Although CO_2 and N_2O emissions typically receive the most publicity regarding their contributions to climate change, the effects of CH_4 should not be over-looked. Methane contributes roughly 20% of the estimated radiative forcing derived from anthropogenic sources. This is second only to CO_2, which is responsible for 60% of estimated anthropogenic radiative forcing (Lassey 2007). In the context of climate change, forcing is defined as changes in the radiation balance of the surface troposphere system imposed by external factors (IPCC 2001). Further, the IPCC indicated in their Fourth Assessment Report (2007b) that CH_4 has a global warming potential (GWP) of 25. This indicates that CH_4 is 25 times more effective at trapping heat in the atmosphere when compared to CO_2 over a 100 year time span. Once emitted, CH_4's lifespan within the atmosphere is approximately 12 years (IPCC 2007b). Chemical reactions with hydroxyl radicals (OH) in the troposphere, producing CH_3 and water as end products, serve as the main mechanism or "sink" for removing CH_4 from the earth's atmosphere (US EPA 2006). Methane's relatively short atmospheric lifetime, coupled with its potency, make it an excellent candidate for climate change mitigation strategies.

2.1.5 Consequences of Climate Change

Increasing concentrations of greenhouse gases within the atmosphere and their subsequent enhancement of the natural greenhouse effect is not without consequence. Global surface temperatures between the years of 1995 to 2006 rank among the 12 warmest years in the instrumental record of surface temperatures (IPCC 2007c). It is estimated that by the year 2030 the average global temperature will have risen 0.5 to 2.5˚C (Moss et al. 2000). A warmer climate will instigate the melting of glaciers, ice caps and ice sheets contributing to an overall increase in global average sea level. Further to

the above, a warmer climate will induce a shift in the world-wide distribution of deserts and wetlands, thus, altering the present-day water supply. It can also be expected that the range and number of pests that affect plants as well as diseases that affect human and animal health will change (Moss et al. 2000).

2.1.6 Legislation Regarding Climate Change

The Kyoto Protocol, a treaty negotiated in 1997, is an agreement under which industrialized countries pledged to reduce their collective emissions of greenhouse gases to 5.2% below 1990 levels over a 5 year period (2008-2012). Not all countries were treated equally under Kyoto. Canada for instance, committed to reducing its greenhouse gas emissions to 6% below 1990 levels by the year 2012 (CBC News 2007). In early 2006 however, Canadian Government officials announced that Canada had no chance of meeting its target under the Kyoto Protocol. Rather, Canada would commit to a self-developed action plan entitled "Turning the Corner". The plan outlined a national target of an absolute 20% reduction in greenhouse gas emissions from 2006 levels by 2020. Individual provinces within Canada have independently committed to targets promising reductions of as much as 300 megatonnes of greenhouse gases by 2020 (Environment Canada 2008). Manitoba, in particular, has remained a supporter of Canada's previous commitment to the Kyoto Protocol (The Legislative Assembly of Manitoba, Bill 15). As a result, Manitoba is committed to reducing its greenhouse gas emissions 6% below its 1990 baseline level (18.0 megatonnes CO_2 equivalent) by 2012.

2.2 Ruminants and Methane

2.2.1 Global Production Estimates

In 2007, the IPCC concluded that agriculture is accountable for 10 to 12% of total global anthropogenic emissions of greenhouse gases (IPCC 2007a). This percentage is equivalent to an estimated production value ranging between 5.1 and 6.1 $GtCO_2$-eq $year^{-1}$. Of this value, CH_4 contributes 3.3 $GtCO_2$-eq $year^{-1}$. As such, agriculture is responsible for roughly 50% of global anthropogenic CH_4 emissions (IPCC 2007a). Farmed ruminant animals are a significant contributor to agriculture's global CH_4 inventory. It is estimated that ruminants contribute approximately 80 Tg of CH_4 $year^{-1}$ (Lassey 2007).

2.2.2 Canadian Production Estimates

According to Canada's 2006 Greenhouse Gas Inventory Report (Environment Canada 2008), the agriculture sector contributed 8.6% of Canada's total greenhouse gas emissions. More specifically, by way of enteric fermentation, animals provided 38.7% of the agricultural sector's total greenhouse gas emissions and 3.3% of Canada's total emissions. Since 1990, greenhouse gas emissions as a result of enteric fermentation have risen by 34.4% (Environment Canada 2008). This is mostly due to an increase in Canada's livestock population. Janzen et al. (1999) reported that Canadian cattle are responsible for the majority of CH_4 emissions from livestock via enteric fermentation. Roughly 72% of total CH_4 emissions from enteric fermentation originate from beef cattle and 25% of CH_4 emissions are attributed to dairy cattle (Janzen et al. 1999).

2.2.3 Benefits of Decreasing Methane Emissions from Ruminants

It is anticipated that global agricultural greenhouse gas emissions will increase in the coming years due to greater demands for food and shifts in diet (IPCC 2007a).

Attempts to reduce CH_4 emissions as a result of enteric fermentation contribute toward the global effort to stabilize atmospheric CH_4. All endeavors directed towards decreasing the concentration of greenhouse gases in the atmosphere, thus reducing climate change, are of social and economic value. From a beef industry perspective, the high costs associated with feeding cattle for production means that profitability depends on the efficient and productive use of feed for maintenance and growth with minimal excesses and losses. Cattle typically lose from 3 to 12% of their ingested energy as eructated CH_4 (Johnson and Johnson 1995). Providing it does not result in a decrease in animal performance, lowering CH_4 loss from enteric fermentation would increase feed efficiency leading to cost reductions and improved production efficiency.

2.3 Methane Production from Microbial Fermentation

2.3.1 Methanogens

Methanogens are a diverse group of microorganisms that belong to the domain Archaea and fall within the kingdom Euryarchaeota (Woese et al. 1990). Phylogenomic analysis has demonstrated that all methanogens posses 31 unique proteins. This discovery indicates methanogenic archaea form a monophyletic group exclusive of all other archaea which likely evolved from *Archaeoglobus* (Gao and Gupta 2007). Methanogens also possess three coenzymes (coenzyme 420, coenzyme M and factor B) that are absent in all other microorganisms (Jones et al. 1987). Coenzymes 420 and M are involved in electron transfer (in place of ferredoxin) and methyl transfer. Factor B is involved in the enzymatic formation of CH_4 from the methyl coenzyme M (Jones et al. 1987). Most importantly, methanogens distinguish themselves from other organisms in that they

predominately derive their metabolic energy from the reduction of CO_2 by hydrogen (H_2) to produce CH_4 (Ohene-Adjei et al. 2007).

2.3.2 Methanogen Habitats

Methanogens only survive in environments with a redox potential below -300 mV (Stewart and Bryant 1988). As a result, they have been located within a wide variety of habitats including salt lakes, bogs, landfills, and the digestive tracts of termites and animals, specifically ruminants such as cattle.

2.3.3 Rumen Methanogens

Organisms from the domain Archaea contribute between 0.3 and 3.0% of the 16S and 18S rRNA within the rumen (Ziemer et al. 2000). In addition, Yanagita et al. (2000) found that 2.8 to 4.0% of ruminal microorganisms displayed an autofloresence under ultra-violet microscopy characteristic of the methanogenic coenzyme 420. These findings signify that a large portion of the archael community within the rumen is comprised of methanogens. Five species of methanogens were reported as isolated from the bovine rumen by McAllister et al. (1996). The five species include *Methanobrevibacter ruminantium, Methanosarcina bakeri, Methanosarcina mazei, Methanobacterium formicum* and *Methanomicrobium mobile*. Of the five species, *Methanobrevibacter ruminantium* and *Methanosarcina bakeri* predominated, with populations exceeding 10^6 mL^{-1}. Unfortunately, cultivation-based studies are unable to uncover the entire extent of rumen microbial diversity, as, not only is it difficult to culture certain species, but a single cultivation-based study is not large enough to give sufficient insight into a community's complete structure (Janssen and Kirs 2008). In recent years, the use of newer molecular-based methods has facilitated the discovery of rumen organisms that differ significantly from those identified via culture techniques. For example, Whiford et al. (2001)

discovered a group of unknown methanogens from the bovine rumen via a method involving polymerase chain reaction (PCR) amplification, cloning and sequencing. Such new discoveries indicate that much work is left to be done in terms of rumen microbial community analysis.

2.3.4 Substrates of Rumen Methanogenesis

The production of CH_4 from organic matter in the rumen is a complex pathway involving a multitude of microorganisms. In fact, methanogens do not become directly involved in the pathway until the final stage of CH_4 production. Primary digestive microorganisms such as bacteria, protozoa and fungi, function to break down proteins, starches and plant cell-wall polymers consumed by the host. As a result, amino acids and simple sugars are formed. These amino acids and sugars are then fermented by primary and secondary digestive microorganisms to produce volatile fatty acids (VFA), H_2 and CO_2. Acetic (C_2), propionic (C_3) and butyric (C_4) acids are the principle VFA generated and as a collective provide the majority of the ruminant animal's carbon and energy needs (Miller 1991).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 \text{ (acetate)} + 2CO_2 + 8H$$

$$C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2 \text{ (propionate)} + 2H_2O$$

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 \text{ (butyrate)} + 2CO_2 + 4H$$

Methanogens then reduce CO_2 to CH_4 in a process that utilizes H_2 (80%) and formate (18%) as the predominant substrates (McAllister et al. 1996). Methane production can be calculated from the stoichiometry of the main VFA formed during fermentation as follows: $CH_4 = 0.45\ C_2 – 0.275C_3 + 0.40\ C_4$, assuming that the amount of H_2 produced is equal to H_2 used, and the recovery rate of H_2 is 90% (Moss et al. 2000). Acetate and butyrate promote CH_4 production, where as propionate formation acts as a competitive

pathway for H_2 use in the rumen. In some cases, methanogens such as *Methanosarcina* can use methylamines, methanol or acetate as alternative substrates for CH_4 formation (McAllister et al. 1996). Unfortunately, CH_4 gas possesses no nutritional value and is viewed as a major inefficiency in ruminant animals.

2.3.5 Biochemistry of Rumen Methanogenesis

As described above, methanogens posses the unique ability to reduce CO_2 to CH_4. This process occurs via four reductive intermediates involving formyl, methenyl, methylene and lastly methyl groups. To date, researchers have identified and determined the structures of six coenzymes that are involved in the reductive process from CO_2 to CH_4. These enzymes include methanofuran (MFR), tetrahydromethanopterin (H4MPT), cofactor 420, cofactor 430, coenzyme M (CoM) and 7-mercaptoheptanoylthreonine (HS-HTP) (DiMarco et al. 1990). Rouviere and Wolfe (1988) best describe the reductive process as a series of seven reactions. The reactions are as follows: CO_2 is fixed using MFR to produce formyl-MFR (reaction 1). Next, the formyl group is transferred to H4MPT via a type of formyltransferase (reaction 2). The formyl group is then converted to a methenyl group using the enzyme 5,10-methenyl-H4MPT cyclohydrolase (reaction 3). Subsequently, the reduced deazaflavin, coenzyme F420 assists in the reduction of methenyl-H4MPT to methylenyl-H4MPT (reaction 4) and of methylenyl-H4MPT to methyl-H4MPT (reaction 5). Thereafter, the methyl group of methyl-H4MPT is transferred to CoM (reaction 6). Lastly, methyl-CoM is reduced to CH_4 by methyl-coenzyme reductase which is a complex system of proteins and cofactors including cofactor 430 (reaction 7). The final reaction completes the cycle and is linked to the activation of CO_2 to form formyl-MFR which starts the process once again.

2.3.6 Associations Between Methanogens and Other Rumen Bacteria

Although CH_4 gas production in ruminants is often viewed negatively due to its lack of nutritional value and subsequent loss of dietary energy, the process does serve a purpose. In the rumen, CH_4 formation is largely responsible for the removal of H_2 as illustrated by Moss et al. (2000) with the following reaction: $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$. The collaboration between fermentative species (H_2 producers) and methanogens (H_2 users) is often referred to as interspecies H_2 transfer. As a result, H_2 levels remain at trace quantities within the rumen environment (Hungate 1966). Research suggests that H_2 concentration in the rumen has a direct effect on fermentative bacteria and how they function. *Ruminococus albus*, a predominant rumen cellulolytic species, produces ethanol, acetate, H_2 and CO_2 when grown in monoculture. Interestingly, this species does not produce ethanol in the rumen nor when cultured together with a methanogen. It is believed that *R. albus* produces H_2 in two distinct ways. The first involves oxidizing pyruvate to acetyl-SCoA, H_2 and CO_2. This reaction is not H_2 sensitive. The second oxidizes NADH to NAD and H_2. This particular reaction is inhibited by high concentrations of H_2. When H_2 is removed from the rumen by methanogens, H_2 is produced from NADH. Consequentially, acetyl-SCoA is transformed to acetate rather than ethanol and ATP yield is enhanced. Increasing levels of ATP can be used for processes such as the production of fibrolytic enzymes, which improve the extent of fiber digestion in the rumen. In addition to their relationship with cellulolytic bacteria, methanogens have been affiliated with several other rumen micro-organisms. These organisms include non-cellulolytic bacteria (Chen and Wolin 1977) such as *Selenomonas ruminantium*, fungi (Marvin-Sikkema et al. 1990) and protozoa (Finlay et al. 1994). Rumen fungi and protozoa produce significant quantities of H_2 from organelles called

hydrogenosomes. Close proximity of methanogens to these organelles facilitates H_2 transfer resulting in CH_4 production. Studies have employed molecular techniques in an effort to quantify the actual percentage of rumen methanogens that are closely associated with protozoa. Sharp et al. (1998) concluded that members of the *methanobacteriaceae* family represent roughly 99% of total methanogens associated with protozoa in the bovine rumen. Meanwhile, free-living *methanobacteriales* comprise only 0.05% of the protozoal fraction. Accordingly, it appears safe to assume that specific methanogen populations may be associated with specific protozoal populations in the rumen (Ohene-Adjei et al. 2007). A better understanding of the relationships methanogens have established with other rumen organisms may provide insight for the development of new or improved CH_4 mitigation strategies.

2.4 Factors Influencing Enteric Methane Production

2.4.1 Diet Quality

Diet quality has a significant effect on CH_4 production from ruminant animals. Feed digestibility (Johnson and Johnson 1995), carbohydrate type (structural vs. non-structural) (Hegarty and Gerdes 1998) and protein content (Ominski et al. 2006) have been hypothesized to influence the extent of CH_4 formation within the rumen. Johnson and Johnson (1995) indicate that as the digestibility of ruminant diets increase, CH_4 losses become more variable. This is due to two primary mechanisms. The first mechanism influences the balance between the rate of carbohydrate fermentation and passage from the rumen. Fermentation of structural carbohydrates is associated with greater losses of CH_4 as a percent of gross energy intake (%GEI) when compared to the fermentation of starch. This is because structural carbohydrates take longer to ferment and have a slow

rate of passage from the rumen (Hegarty and Gerdes 1998). The second mechanism controls the available H_2 supply for CH_4 production via the ratios of VFA produced. High proportions of acetic acid relative to propionic acid in the rumen result in increased CH_4 formation. Structural carbohydrate fermentation results in a higher acetic: propionic acid ratio where-as starch fermentation favors greater levels of propionic acid production (Hegarty and Gerdes 1998).

In determining the quality of a particular diet or feedstuff, protein content is often examined. Low quality forages are characterized as having low concentrations of crude protein (CP) and high concentrations of neutral detergent fibers (NDF). In a grazing trial by Ominski et al. (2006), backgrounding animals experienced greatest CH_4 losses (11.3 %GEI) when pasture conditions were characterized by low quality forage. The authors attributed the intensive CH_4 production to less than optimal rumen fermentation resulting from insufficient forage CP.

2.4.2 Forage Preservation (Silage vs. Hay)

Method of forage preservation is believed to have an effect on CH_4 production but information on the subject is limited. Sundstøl (1981) indicated that ensiling rather than drying forages would effectively decrease CH_4 production as a %GEI. The rationale behind this theory is that extensive fermentation involved in the ensiling process will decrease digestion in the rumen and consequently decrease CH_4 formation. Mechanistic modeling conducted by Benchaar et al. (2001) supports the findings of Sundstøl. Total CH_4 production (Mcal d^{-1}) was depressed by 33% when alfalfa silage was used as opposed to alfalfa hay. It must be noted that the forage's stage of maturity at the time of harvest/preservation will have an effect on CH_4 production. Methane production per unit of intake in ruminants tends to be positively correlated with the maturity of the forages fed

(McAllister et al. 1996). This association is attributed to a reduction of forage quality with age.

2.4.3 Level of Feed Intake

Increased levels of feed intake are associated with a decrease in the loss of CH_4 as a %GEI. In fact, Johnson and Johnson (1995) indicate that CH_4 as a %GEI decreases by nearly 1.6% for each increased increment of feed intake. The effect of feeding level on CH_4 emissions is most easily explained by its direct effect on the passage rate of feed particles from the rumen. Increased levels of feed intake stimulate an increased rate of particle passage. Particles that pass quickly through the rumen experience less extensive fermentation since their exposure to rumen micro-organisms is significantly reduced. In addition to decreased fermentation, rapid rate of passage also favors propionate production which is an alternative pathway for the use of H_2 (Mathison et al. 1998). Thus, feeding diets rich in available carbohydrates, such as grains, at limited intakes will result in high fractional CH_4 losses. Conversely, feeding the same diet at higher levels will result in low fractional CH_4 losses (Johnson and Johnson 1995). It should be noted that diets consisting entirely of roughage do not affect rate of passage to the same extent as concentrate or mixed diets (Mathison et al. 1998). Beauchemin and McGinn (2006a) conclude that feeding cattle for maximum weight gain is an effective CH_4 mitigation strategy for the cattle industry. This is because it reduces the proportion of feed energy lost as CH_4 each day while reducing overall days to market.

2.4.4 Temperature

Canada's climate is incredibly diverse. In summer months temperatures regularly surpass 30˚C. Throughout the winter however, most daytime readings are almost always well below freezing. Beef cattle raised in cold environments typically experience

heightened maintenance demands and as a result increase their feed intake. Increased feed intake is associated with increased rate of particle passage through the rumen which in turn is correlated with decreased CH_4 production (McAllister et al. 1996). Kennedy and Milligan (1978) reported that ruminal passage rate constants of fluid and particulate matter increased by roughly 54 and 68% in cold-adapted sheep. These increases were accompanied by a 30% depression in CH_4 production. The authors also detected a decreased acetate/propionate ratio in cold acclimated sheep. They suggest that the decreased ratio may indicate a shift towards propionate production in the rumen contributing to reduced CH_4 formation. Contrasting evidence suggests that decreased CH_4 production may not always be associated with colder temperatures. Research by Von Keyerslingk and Mathison (1993) showed that CH_4 emissions were 25% higher from sheep housed at 4.7˚C than in those housed at 21˚C. When CH_4 was expressed as a percent of digestible energy, 14% more CH_4 was produced in the cold environment. This increase may be attributed to a combination of two factors: 1) a substantial increase in dry matter (DM) intake at cold temperatures and 2) the sheep were not sufficiently cold stressed. In general, information relating to temperature's effect on CH_4 formation from ruminants, particularly beef cattle, is limited. Further insight into this topic would be of value to producers in countries such as Canada where temperature fluctuates on a seasonal basis.

2.4.5 Genetic Selection

Selection of more feed efficient animals based on estimated breeding values is a novel approach to CH_4 mitigation employed by the beef industry. Beef cattle express significant variation in both genotypic and phenotypic traits relating to feed efficiency. An example of one of these traits is residual feed intake (RFI), which is the difference

between an animal's actual feed intake and its expected intake based on body weight and growth rate over a specified period (Nkrumah et al. 2006). Low RFI beef cattle, as described by Hegarty et al. 2007, eat less than expected for their body weight and average daily gain (ADG). In cattle, RFI has a moderate heritability (h^2 = 0.39) and is correlated with compositional traits such as back-fat depth (Arthur et al. 2001). With the exception of highly digestible diets, CH_4 formation is positively correlated with energy intake. Thus, CH_4 emissions should be reflective of greater intakes since more substrate is available for fermentation and consequently more H_2 is supplied for utilization by rumen methanogens. As a result, selecting livestock for lower feed intakes should offer a direct strategy for decreasing CH_4 emissions from beef cattle (Hegarty et al. 2007). Nkrumah et al. (2006) were the first to conduct a trial indicating significant differences in CH_4 emissions among animals differing in RFI. Data from the study showed that CH_4 production was 28 and 24% less in low-RFI animals than in high- or medium-RFI animals. A subsequent study by Hegarty et al. (2007) confirmed the findings of Nkrumah et al. (2006). Low RFI cattle in this trial experienced an 18 g d^{-1} decrease in CH_4 production rate. Aside from level of intake, it is unknown what mechanisms are responsible for the observed differences in CH_4 production among individual cattle. It is thought that the decrease in formation could be attributed to differences in metabolizability. A different theory suggests that diet is less important as individual animals simply have different CH_4 formation capabilities. Hackstein et al. (1996) proposed a genetic link between the methanogen and its host. This indicates that the presence of methanogens is of a genetic nature rather than a dietary one. In any case, selecting animals based on estimated breeding values for RFI is an effective and practical strategy for reducing CH_4 emissions from the beef cattle sector. Predictive equations

suggest the greatest diminution will be achieved on low digestibility diets (Hegarty et al. 2007).

2.4.6 Probiotics

The term probiotic meaning "for life" is currently used to describe live microorganisms, which when consumed in adequate amounts confer a health effect on humans and animals (Food and Agriculture Organization of the United Nations (FAO) 2001). There are several publications that imply the addition of microbial feed additives to ruminant diets may have an effect on enteric CH_4 production, however, results from trials in which probiotics have been used are often conflicting and lack consistency. Miller-Webster et al. (2002) demonstrated that including yeast culture products (YC1, Diamond-V XP and YC2, A-Max) in a continuous culture system increased DM digestion and propionic acid production while decreasing protein digestion and acetic acid production. As mentioned earlier in this review, lower acetic:propionic acid ratios correlate with reduced CH_4 production in the rumen. Conversely, Chiquette and Benchaar (1998) found no effect on the molar proportions of ruminal VFA when a combination of *Saccaromyces cerevisiae* and *Aspergillus oryzae* were added to the diet of dairy heifers. A unique trial by Mwenya et al. (2004) explored the effects of blending prebiotic (galacto-oligosacharides) and probiotic (vitacogen) additives in the diet of Holstein dairy cows on enteric CH_4 production. Interestingly, the diet containing galacto-oligosacharides led to an 11% decrease in CH_4 emissions (L d^{-1}). However when the prebiotic was supplemented together with probiotic, CH_4 emissions increased by 17.5%. It should be noted that probiotic supplementation did trigger an unexplainable increase in live weight gain. Consequently, CH_4 emissions expressed per unit of production (g kg^{-1} live weight gain) from animals fed a probiotic-supplemented diet were 2.4 fold lower than in the

control diet. The variable responses of cattle to probiotics have led producers to be skeptical about the benefits of including them in feeding programs (Moss et al. 2000). Further research is required to establish the potential of probiotics for reducing CH_4 production *in vivo* (Boadi et al. 2004).

2.4.7 Essential Oils

Recent interest has been expressed in utilizing natural feed additives to alter ruminal fermentation and lower CH_4 emissions. Essential oils are plant secondary metabolites that are responsible for the odor and color of plants and spices (Castillejos et al. 2005). They have traditionally been used for their pleasant fragrance, taste, preservative and antiseptic properties. Essential oils are comprised mainly of monoterpenes as well as cyclic hydrocarbons and their alcohol, aldehyde or ester derivatives (Wallace 2004). They are typically obtained from plants through steam and/or water distillation (Losa 2001). Examples of some very widely used essential oils include thymol, limonene and guaiacol. Thymol is derived primarily from thyme and oregano, whereas limonene is abundant in citrus peel oil and guaiacol is a large component of clove oil (Castillejos et al. 2005). Essential oils are known to possess antimicrobial properties and manipulate fermentation in ruminant animals by selective modulation of certain microbial species. Evans and Martin (2000) found that the addition of thymol, at a concentration of 400 μg mL^{-1}, reduced the total concentration of VFA in 24 h *in vitro* incubations of ruminal fluid. They concluded that thymol was a strong inhibitor of glucose fermentation by *Streptococcus bovis* and *S. ruminantium* resulting in decreased lactate and CH_4 production. In an *in vivo* study by Beauchemin and McGinn (2006b), an essential oil and spice extract (Crina Ruminants; Azko Nobel Surface Chemistry S.A., Cedex France) was incorporated into the diet of spayed heifers at a concentration of

1 g d^{-1}. They observed no effect on the concentration or proportion of VFA, or on total daily CH_4 production. To date, only a modest amount of research has been conducted to determine the effects of essential oils on methanogens. From this work however, researchers are convinced that inclusion of essential oils into ruminant diets may serve as a strategy for targeting rumen methanogenic populations as they possess specific antimicrobial activity (Beauchemin and McGinn 2006b). Further research is required to determine appropriate level of inclusion into animal diets, to identify the actual methanogenic bacteria affected and to establish the longevity of the effects on the methanogen populations.

2.4.8 Saponins

Saponins are high molecular weight (MW) glycosides consisting of sugars linked to either a steroidal or triterpene moiety. They occur naturally within several temperate plants including alfalfa, oats and daisies. Saponins are unique compounds in that they have the ability to foam and it is for this reason, that they have been employed as a natural detergent for centuries (Wallace 2004). When consumed by ruminants, saponins affect fermentation by suppressing protozoa and selectively inhibiting some bacteria (Cheeke 2000). Research has confirmed that a strong symbiotic relationship exists between methanogens and protozoa (Finlay et al. 1994). From this knowledge, it is fair to propose that the selective suppression of protozoa may lead to a reduction of CH_4 formation and increased efficiency of feed utilization by ruminants (Dohme et al. 1999). The deleterious effect of saponins on protozoa is attributed to their ability to bind to sterols within protozoal cell membranes and cause cell lysis. Since protozoa are the natural predators of ruminal bacteria, it is expected that their decline will cause bacterial numbers to flourish. Saponins are also selective against some bacteria, most noticeably those that are gram-

positive, for example, *S. bovis*. Subsequently, rumen bacteria may increase in number but decrease in diversity (Mathieu et al. 1996). Hess et al. (2003) found the fruit of *S. saponaria*, reduced CH_4 production by 11% in an *in vitro* culture of grass and legume-supplemented diets. A more recent trial by Goel et al. (2008) utilizing Fenugreek and *Sesbania in vitro* produced conflicting results. These authors discovered that a decrease in protozoal number was not accompanied by a decrease in CH_4 production in incubations using rumen liquor from hay-fed animals. Further research is required to determine the effects of dietary supplementation of plant saponins on ruminant CH_4 production.

2.4.9 Organic Acids

In recent years, the usefulness of dicarboxylic acids (particularly fumarate and malate) as natural feed additives in ruminant diets has been examined. It is proposed that organic acids decrease methanogenesis and the energy losses associated with CH_4 in the rumen. Fumaric acid is a metabolic precursor of propionate whose production provides an effective sink for H_2, thus diverting it from methanogenesis (Asanuma et al. 1999). *In vitro* studies have also indicated that fumarate reduces CH_4 production by stimulating the proliferation of cellulolytic bacteria and the digestion of fiber (Beauchemin and McGinn 2006b). Lopez et al. (1999) observed that adding sodium fumarate at concentrations ranging between 0 and 10 m*M* in a hay-barley grain diet decreased CH_4 production by 5% in batch culture. These results were later confirmed using a Rustitec to simulate the actual rumen environment. Likewise, Carro and Ranilla (2003) reported that adding fumarate at the same concentrations used by Lopez et al. (1999) to various feed grains in batch culture increased VFA concentrations, decreased the acetate:propionate ratio and reduced CH_4 production by nearly 5%. Unfortunately, the same success has not been demonstrated by *in vivo* studies. A trial by Beauchemin and McGinn (2006b), added 29 g

kg DMI^{-1} of fumaric acid to a high forage diet typical of backgrounded cattle in western Canada. Although they observed a decrease in the acetate:propionate ratio consistent with the *in vitro* studies described above, they did not report a decrease in CH_4 production. It is possible that organic acids may not be capable of mitigating CH_4 production in the rumen environment due to confounding factors. Further research is required in this area before organic acids are recommended for use in the beef industry.

2.5 Condensed Tannins and Rumen Methanogenesis

2.5.1 Condensed Tannins

Condensed tannins (CT), also known as proanthocyanidins, are a plant polyphenol consisting of polymerized flavan-3-ol units. Condensed tannins are highly capable of binding with proteins and it is this ability which leads to their extensive use as a leather tanning reagent. It is believed that plants first developed CT as a defensive strategy against invasion from pathogenic microorganisms which later evolved as a means to protect themselves from being eaten by insects and grazing herbivores (Barry 1989). The chemistry of CT is multifaceted which means that individual complexes can exhibit extensive differences in structure. The differences can be attributed to variation in the hydroxylation of the B-ring in the flavan-3-ol monomer units, as well as the stereochemistry of the heterocyclic C-rings which can take on either a cis or trans formation and ultimately determines the attachment of the monomeric units to one another. In addition to this, interflavonoid linkages of monomeric units can fluctuate (C4/C8 or C4/C6) which affects the shape of the polymer chain. Lastly, there is variation in the actual number of monomer units within each polymer which ultimately determines its weight (Barry and McNabb 1999). It is important to acknowledge the diverse

structures of individual CT as these differences are accountable for the changing biological properties associated with them.

2.5.2 Location of Condensed Tannins

Condensed tannins have a broad distribution in nature and have been extracted from plants (trees, shrubs and legumes) at various locations around the globe. Within western Canada, CT-containing plants that are of interest to the cattle industry for inclusion into livestock diets are mainly legumes such as *Onobrychis viciifolia* (sainfoin) and *Lotus corniculatus* (birdsfoot trefoil). In a plant, CT are stored within cell vacuoles located in stems, bark, leaves, flowers or seeds (Barry 1989). In numerous forage species, CT are localized within specific tissues. In white clover, CT are found solely in the flower (Jones et al. 1976) whereas in alfalfa they are located exclusively in the seed coat (Koupai-Abyazani et al. 1993). In other legumes, such as sainfoin, CT occur in all organs except for the cotyledons and roots, with the highest concentrations occurring in the leaves (Lees 1993). Research has indicated that the concentration of plant CT varies considerably and is dependent upon forage species, cultivar type, stage of plant development, soil fertility and other environmental factors such as the season in which they are grown and harvested. Warmer temperatures are believed to induce increased CT formation, particularly in plant species that naturally contain high levels of tannin. A study by Gebrehiwot et al. (2002) utilizing *Lotus* species, found mean herbage CT concentrations from spring and summer harvested plants nearly doubled as compared to those measured in the fall. This data supports work by Lees et al. (1994) where clones of *L. uliginosus* (synonymous with *L. pedunculatus*) produced proportionately higher levels of CT when grown at 30˚C versus 20˚C.

2.5.3 Beneficial Effects of Condensed Tannins on Ruminant Performance

Condensed tannins can be beneficial or detrimental to ruminant animals depending on level of inclusion within the diet. Concentrations ranging between 2-4% of DM content have led to beneficial effects on animal performance (Aerts et al. 1999). As previously mentioned, CT have the unique ability to bind with proteins. This process is pH dependent and occurs in the rumen (pH, 6.0-7.0), resulting in the formation of stable complexes between CT and proteins, thereby reducing rumen proteolysis. Once past the rumen, the lower pH of the abomasum (2.5-3.5) dissociates the CT-protein complexes resulting in enhanced digestion and absorption of essential amino acids in the small intestine (Aerts et al. 1999). Greater efficiency of protein utilization has been connected to better growth rates in backgrounding cattle. Marten and Ehle (1984) found that heifers grazing sainfoin and birdsfoot trefoil had higher live weight gains than heifers grazing alfalfa. In addition, reducing the quantity of soluble protein in the rumen limits the production of the stable foam that is associated with bloat. Bloat is a challenge since mild forms can decrease animal feed intake and if left undetected, quickly result in death (Tanner et al.1995). Condensed tannin-containing forages also have considerable potential for parasite control. Several trials have demonstrated reductions in intestinal parasites in sheep (Niezen et al. 1995) and deer (Hoskin et al. 2000) fed CT-containing forages. However, information examining the effect of tannin-containing diets on intestinal parasite infection in cattle is limited (Waghorn and McNabb 2003).

2.5.4 Negative Effects of Condensed Tannins on Ruminant Performance

At high CT concentrations, the plant's defensive characteristics become apparent. Accordingly, feeding CT at concentrations in excess of 6% DM in ruminant diets has demonstrated negative effects on animal productivity. Tannins are astringent-tasting

compounds that can decrease the palatability of forages. They also cause dry-mouth and decrease voluntary intake by forming complexes with salivary glycoproteins (Goel et al. 2005). Lower rates of digestion (higher rumen fill) associated with the presence of CT in the diet can also contribute to reduced intake (Makkar et al. 1995). Further to the above, the actual nutritional value of feedstuffs can be negatively affected in the presence of CT. Digestibility appears to decline with increasing CT concentration in the diet. Decreased digestibility occurs as a consequence of complexes formed between CT and cell-wall polysaccharides as well as bacterial enzymes (Reed 1995). Condensed tannins also have an unfavorable impact on nutrition through their ability to complex metal ions, thus reducing the bioavailability of essential micronutrients such as iron, zinc and sulfur. Lower voluntary feed intakes compounded with diminished nutritive value of the diet result in decreased live weight gain and productivity in ruminants (Waghorn and McNabb 2003). Animals such as mule deer, rats, giraffes and goats (to a limited extent) secrete proline rich proteins in their saliva which bind to and reduce negative effects of CT. Unfortunately, this mechanism is not present in cattle.

2.5.5 Effect of Condensed Tannins on Rumen Microorganisms

Condensed tannins are generally regarded as inhibitory to the growth of rumen microorganisms. The basis of this effect is believed to stem from their ability to complex with polymers such as proteins and cellulose as well as with minerals. Rumen bacteria can be directly inhibited by CT interacting with membranes, cell walls and/or extracellular proteins. They may also be indirectly inhibited via nutrient deprivation (Smith et al. 2005). Jones et al. (1994) found that CT from sainfoin bound to cells in four strains of proteolytic ruminal bacteria: *Butryvibrio fibrisolvens* A38, *S. bovis* 45S1, *Prevotella ruminicola* B_14, and *Ruminobacter amylophilus* WP225. In the presence of 25 µg of CT

mL^{-1}, total protease activity of *B. fibrisolvens* and *S .bovis* was reduced by 48 and 92% respectively. These bacterial strains also experienced suppressed growth in addition to morphological changes. Condensed tannins did not inhibit protease activity or growth of *R. amylophilus* under concentrations of 100 µg mL^{-1}. Conversely, neither growth nor protease activity of *P. ruminocola* was inhibited at any CT concentration tested up to 300 µg mg of $cells^{-1}$. *Butyrivibrio fibrosolvens* and *S. bovis* are both characteristically gram-positive bacteria. In spite of the complex formation of their cell wall polymers, CT were able to penetrate the cell wall and react with one or more ultrastructural components to selectively inhibit cell wall synthesis. Decreased proteolytic activity is likely caused by hindered export of proteases from the cell in the presence of CT. Other researchers (Smith et al. 2004) reported a shift in predominance of gram-positive bacteria to gram-negative bacteria in the presence of CT. Cellulolytic bacteria, which are essential for fiber digestion, also appear to be CT sensitive. McSweeney et al. (1998) showed that the presence of Calliandra tannins at a concentration of 2-3% in the diet markedly decreased populations of *Fibrobacter succinogenes* and *Ruminococcus* species. Bacteria that are predominant in CT-rich systems may not necessarily be resistant to CT, rather they are better able to cope or have a greater ability to access limited nutrients (Smith et al. 2005).

2.5.6 Effect of Condensed Tannins on Enteric Methane Emissions

Research has indicated that feeding tannin containing forages to ruminant animals may reduce enteric CH_4 production (Carulla et al. 2005). It is believed that CT may affect rumen methanogenesis via two distinct mechanisms. The first is an indirect affect caused by a reduction in fiber digestion which leads to decreased H_2 production in the rumen. The second is a direct affect caused by inhibiting the growth of methanogens (Tavendale et al. 2005). Carulla et al. (2005) examined the affects of including roughly 25 g of CT

(extracted from the bark of *Acacia mearnsii*) kg DM^{-1} in a diet fed to growing lambs. In their investigation, tannin supplementation suppressed NDF and ADF digestibilities. The reduced fiber digestion was associated with a shift in the molar proportions of VFA from acetate to propionate at a constant total VFA concentration in the rumen fluid. More importantly, CH_4 emissions decreased by 13%. These results concur with data from Woodward et al. (2001) who demonstrated that feeding *Lotus corniculatus* silage, with a CT concentration of 2.59 g 100 g DM^{-1}, to lactating dairy cattle decreased CH_4 emissions. Lactating dairy cows fed *L. corniculatus* produced 26.90 g CH_4 kg DMI^{-1}, whereas cattle fed ryegrass silage emitted 35.13 g kg DMI^{-1}.

In spite of the above findings, some research conflicts with the hypothesis that CT reduces CH_4 emissions. Beauchemin et al. (2007) supplemented quebracho tannin extract at a concentration up to 2% DM in a forage-based diet fed to growing cattle. In their trial, tannin supplementation did not affect ADF or NDF digestibility. However, it did linearly decrease the apparent digestibility of CP. Further, increased levels of tannin supplementation linearly decreased total VFA concentration in the rumen, in addition to decreasing the molar proportion of acetate resulting in a decreased acetate:propionate ratio. Interestingly, there were no effects of quebracho tannin extract supplementation on CH_4 emissions, regardless of how emissions were expressed. There is still considerable uncertainty regarding the effectiveness of CT's ability to reduce CH_4 emissions from cattle. Beauchemin et al. (2007) suggest that many studies reporting decreased CH_4 production from ruminant animals may be attributed to the fiber composition of the diet rather than CT. Since low fiber diets are associated with lower CH_4 emissions (Johnson and Johnson 1995) feeding diets differing in nutrient composition may confound trial results. Additionally, CT extracted from different plants vary in their ability to bind with

carbohydrates and protein (McAllister et al. 2005). Therefore, the impact of CT on enteric CH_4 production by ruminants may be highly dependent on the species of plant the CT is extracted from or that is incorporated into the diet. Although several researchers have made reference to the potential affect of CT on the methanogenic population of the rumen, most information is purely speculative. Using molecular techniques to study rumen methanogens would provide a clearer picture of the affect of CT on methanogenesis.

2.5.7 Optimal Concentrations of Condensed Tannins in Ruminant Diets

The effect of CT on animal performance is highly dependent on their ability to complex with proteins. The protein-complexing capabilities of CT are determined by their molecular characteristics, which in turn are specific to the plant species they originate from. Condensed tannins with high MW have been observed to precipitate less protein per unit weight than those with low MW (Barry 1989). Consequently, it is important to have knowledge of both the molecular structure and concentration of CT within plant species intended for ruminant consumption. The MW of CT from specific plant species is consistent and well documented. Sainfoin for example, contains high MW CT (MW 17,000-28,000) whereas *Lotus* species contain low MW CT (MW 6,000-7,000) (Barry 1989). The CT concentration of a plant is highly variable and differs among as well as within plant species. Much of the variation within a plant species is attributed to differences in environmental factors such as temperature and soil fertility. Countries such as New Zealand have significantly researched CT concentrations of native plant species whereas Canada is just beginning this process. Berard et al. (2011) recently examined the extractable CT concentrations in forage legumes grown at eight sites across the prairie region of Western Canada (Berard et al. 2011). *Dalea purpurea* (purple prairie

clover), a native legume, had the highest mean CT concentration of all species examined in the survey at 68.7 g kg^{-1} DM. Sainfoin had the second highest mean CT concentration at 46.0 g kg^{-1} DM with a range of 16.3 to 94.4 g kg^{-1} DM between six different varieties. The species with the third highest CT concentration was birdsfoot trefoil with a mean concentration of 15.1 g kg^{-1} DM and a range of 0.0 to 25.7 g kg^{-1} DM between two varieties. Feeding CT at concentrations ranging between 20-45 g kg^{-1} of forage DM has elicited positive responses in ruminant animal performance (Min et al. 2003). To be most effective, CT with high MW may need to be fed at slightly higher concentrations than CT with low MW (Barry 1989).

Information is limited regarding the effects of Canadian grown CT-containing forages on ruminant CH_4 production. Feeding *L. corniculatus* grown in New Zealand to lactating dairy cattle significantly decreased enteric CH_4 production per kg DMI and per kg milk-solids produced. The dietary concentration of CT within the *Lotus* was approximately 25.9 g kg DM^{-1} (Woodward et al. 2001). Further research is required to determine the naturally occurring level of CT within forages grown throughout different regions of Canada and their subsequent effect on CH_4 production from ruminants.

2.6 Summary

Atmospheric greenhouse gas concentrations have substantially increased since the beginning of the industrial revolution. It is understood that higher greenhouse gas concentrations will enhance the natural greenhouse effect and contribute to an acceleration of global climate change. Warmer global temperatures will instigate increases in global sea level, shifts in world-wide water distribution and alter the geographical distribution of pests/diseases that affect both human and animal health.

Ruminant animals, particularly beef cattle, contribute to Canada's greenhouse gas inventory by emitting CH_4 as a consequence of enteric fermentation. In addition to being an environmental concern, CH_4 from cattle represents an inefficiency of the production system since significant portions of ingested energy are lost as eructated CH_4 gas. Decreasing enteric CH_4 emissions from cattle would increase overall feed efficiency and reduce production costs.

Enteric CH_4 production can be influenced by a multitude of factors. Examples of these factors include diet quality, method of forage preservation, level of intake and inclusion of feed additives. Feeding CT, a type of plant polyphenol, may provide an avenue for reducing rumen methanogenesis. In western Canada, CT-containing plants such as sainfoin and birdsfoot trefoil are of interest to the cattle industry. It has been speculated that CT may indirectly affect methanogenesis by reducing fiber digestion and decreasing H_2 production in the rumen. Further, CT may directly affect methanogenesis by inhibiting rumen methanogens. Feeding CT at concentrations ranging between 20-45 g kg^{-1} of forage DM has elicited positive responses in cattle performance including decreased enteric CH_4 production. The CT concentration of a plant is highly variable and differs between as well as within plant species. Information is limited regarding the naturally occurring levels of CT within forages grown throughout different regions of Canada and their subsequent effect on ruminant CH_4 production. As is the case, further research is required to determine the feasibility of feeding CT-containing plants as a CH_4 mitigation strategy for the Canadian cattle industry.

3.0 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

Enteric methane (CH_4) emissions as a percent gross energy intake (%GEI) from cattle can be decreased by incorporating condensed tannins (CT) into the diet at a concentration ranging between 20-45 g kg^{-1} of forage dry matter (DM). The effect of CT on CH_4 production may be attributed to the inhibition of rumen methanogen populations. It may also be attributed to a reduction in fiber digestion leading to a reduction in hydrogen (H_2) and CH_4 production within the rumen. Feeding silage rather than hay will provide an additional avenue for reducing CH_4 production from cattle. Forages undergo substantial fermentation during the ensiling process which decreases the extent of digestion required in the rumen thus limiting CH_4 formation.

3.2 Objectives

The specific objectives of the present study were:

1) To evaluate the effect of CT in a Manitoba grown forage (sainfoin) on enteric CH_4 emissions and rumen fermentation.

2) To assess the effect of method of feed preservation (silage versus hay) on enteric CH_4 emissions and rumen fermentation.

3) To determine the impact of CT in a Manitoba grown forage (sainfoin) on the rumen bacterial ecology of backgrounding steers.

4) To discern the effects of the following parameters on the methanogenic profile of rumen fluid from backgrounding steers:

    a. Methane production (%GEI)
    b. Dry matter (DM) intake as a percent of body weight (%BW)
    c. Gross energy intake (MJ/d^{-1})
    d. Crude protein intake (kg d^{-1})
    e. CT intake (g d^{-1})
    f. Environmental temperature (˚C)

4.0 MANUSCRIPT

Methane emissions and rumen microbial changes in steers fed condensed tannin containing diets under western Canadian conditions.

4.1 ABSTRACT

Methane (CH_4) emissions from ruminants contribute as much as 3% of the total greenhouse gas emissions in Canada (Matin et al. 2004). Naturally occurring, anti-microbial plant compounds such as condensed tannins (CT) may be used to reduce enteric CH_4 emissions from cattle. We hypothesized that sainfoin, a CT-containing leguminous plant, fed to beef cattle as hay or silage during a western Canadian winter would result in a reduction in CH_4 emissions without compromising animal performance.

Forty yearling beef steers (293.5 $\pm$ 21.9 kg) were fed four diets in a factorial design consisting of two forage species (sainfoin or alfalfa) and two preservation methods (silage or hay). The trial was conducted over 15 weeks (wks) with: two wks of adaptation (alfalfa silage or hay); one wk of adjustment (gradual conversion of half the cattle to sainfoin silage or hay); a nine-wk experimental period (sainfoin or alfalfa silage or hay); and three wks withdrawal (alfalfa silage or hay). Individual animal intake was recorded daily. At the beginning of each sample wk, animal weight, 24-h CH_4 expiration and rumen fluid samples were obtained. Enteric CH_4 emissions were quantified using the sulphur hexafluoride (SF_6) tracer gas technique (Boadi et al. 2002) and rumen fluid samples, acquired via a Geishauser oral probe, were analyzed for volatile fatty acid (VFA) profiles. Rumen methanogen populations were characterized using terminal restriction fragment length polymorphisms (TRFLP) analysis. Specific bacteria, including *Ruminicoccus albus*, *Streptococcus bovis* and *Prevotella ruminicola* were quantified with real-time PCR (RT-PCR).

Sainfoin, which was grown in a Manitoba production environment, contained 1% CT on a dry matter (DM) basis. Sainfoin diets were well-received by the yearling steers

as animals consuming sainfoin silage (SS) and sainfoin hay (SH) experienced DM intakes of 2.5 and 2.8 as a percent of body weight (%BW) respectively. Further, steers receiving the sainfoin silage (SS) and sainfoin hay (SH) diets realized gains of 0.7 and 0.6 kg d^{-1} respectively and did not differ from those animals receiving alfalfa diets. A significant decline ($P < 0.05$) in enteric CH_4 formation could only be detected from SH-fed animals as compared to alfalfa hay (AH) fed animals when CH_4 was expressed as L d^{-1} or L kg BW^{-1} but not as L kg DMI^{-1} or as a %GEI. A decline in CH_4 formation was not detected from SS-fed animals as compared to alfalfa silage (AS). It is possible that the sainfoin diets fed did not contain sufficient quantities of CT to realize reductions in CH_4 to the extent we hypothesized. Feed preserved as hay, particulary in the alfalfa diets, resulted in higher ($P < 0.05$) quantities of total VFA production as well as lower ($P < 0.05$) acetic:propionic acid ratios. Terminal restriction fragment length polymorphism indicated that the rumen archaeal community structure of experimental animals remained stable regardless of diet type or sample wk. Relative abundance data from RT-PCR showed that silage diets had a suppressive effect on structural carbohydrate-fermenting bacteria as well as *Lactobacillus species*. Irrespective of preservation technique, ciliate protozoa populations were enhanced by feeding sainfoin. Methanogens were reduced in the rumen fluid samples of steers fed SS but not SH. It is unlikely that the abundance of methanogens from SS-fed animals decreased as a direct result of CT, but rather a reduction in the flow of hydrogen (H_2) since prokaryotic bacteria were less abundant in the silage diets.

4.2 INTRODUCTION

In recent years, CH_4 gas has gained increased recognition for its role in the greenhouse effect. Methane is second only to carbon dioxide (CO_2) in its contribution of estimated radiative forcing derived from anthropogenic sources (Lassey 2007), and has been designated a global warming potential (GWP) of 25 by the Intergovernmental Panel on Climate Change (Intergovernmental Panel on Climate Change (IPCC) 2007b). Ruminant livestock are considered major contributors to global CH_4 production. Cattle typically lose from 3 to 12% of their ingested energy as eructated CH_4 (Johnson and Johnson 1995). These losses are an environmental concern and represent a loss of metabolizable energy for the animal. Reducing enteric CH_4 emissions from cattle would have a two-fold effect of decreased environmental CH_4 and increased production efficiency.

Feeding CT, a plant polyphenol, has lead to beneficial effects on ruminant performance including reduced enteric CH_4 production when incorporated in the diet at concentrations ranging between 2-4% of DM content (Aerts et al. 1999). Within western Canada, legumes such as *Onobrychis viciifolia* (sainfoin) and *Lotus corniculatus* (birdsfoot trefoil) naturally contain CT and are of interest to the beef cattle industry as potential feedstuffs. Due to the multifaceted chemistry and fluctuating concentrations of CT within plants, further research is required to demonstrate the effects of CT from forages grown in a Canadian production environment on cattle performance.

Method of forage preservation, ensiling versus field drying, is believed to have an effect on enteric CH_4 production as fermentation which occurs during ensiling may decrease digestion in the rumen and reduce CH_4 formation as a %GEI (Sundstøl 1981).

Additional *in vivo* research is required to better understand the outcome of selecting one method of forage preservation over the other on enteric CH_4 formation.

To date, most research examining the effects of enteric CH_4 production by ruminants has been conducted under temperate conditions for relatively short periods of time (four wks). During a western Canadian winter, the ambient temperature can be as low as -40 degrees Celsius (°C). These conditions result in significant behavioral and metabolic changes to cattle that may affect digestive processes and rumen fermentation (Young 1981; 1983). This experiment was designed to assess the effect of CT coupled with forage preservation technique on CH_4 emissions and rumen fermentation during a western Canadian winter over a period of three months.

4.3 MATERIALS AND METHODS

4.3.1 Forage Production

Twenty-acre stands of sainfoin and alfalfa were established near Brandon, Manitoba. Sainfoin and alfalfa stands were harvested at the onset of first bloom in early June. Re-growth from both stands was harvested again, when plants began to bloom, in late July. Sainfoin and alfalfa stands were each preserved as round-bale silage and hay (n = 4 diets). Forage preserved as silage was permitted to wilt in the swath to approximately 50% DM before baling. Shortly thereafter, the high-moisture bales were wrapped in plastic to ensure maximum preservation and to prevent spoilage. Forage preserved as hay, was wilted to roughly 85% DM in the swath and then baled.

4.3.2 Animals and Feeding Strategy

The effect of forage type (sainfoin or alfalfa) and preservation technique (silage or hay) on enteric CH_4 production, rumen fermentation characteristics and rumen bacterial

ecology was examined in 40 yearling steers (293.5 $\pm$ 21.9 kg). Steers were randomly assigned to one of four feedlot pens with an equal total body weight/pen.

The 15-wk feeding trial consisted of four dietary treatments fed over three periods as described in Figure 4.1. Dietary treatments were:

a) Sainfoin Silage (SS)
b) Sainfoin Hay (SH)
c) Alfalfa Silage (AS)
d) Alfalfa Hay (AH)

Period one served as a three-wk adaptation period to ensure that steers were adapted to preservation technique (silage or hay). Steers to be fed either AS or SS were fed AS while steers to be fed either AH or SH were fed AH. Thereafter, wk three served as an adjustment period for forage type. Sainfoin silage or SH was gradually introduced to those steers which were to receive sainfoin. A gradual introduction of sainfoin into steer diets was necessary to prevent a decline in their DM intake. In a preliminary intake trial, dairy heifers refused feed when their AH diet was immediately substituted with a SH diet. During the second experimental period, wks 4-11, steers were fed one of the four dietary treatments described above. In period three, wks 12-15, SS and SH diets were replaced with AS and AH respectively to determine the effects of removal of CT from the diet on the parameters described above.

Steers were fed once daily at approximately 0900. Feed offered was adjusted to ensure 5% orts. Water and mineral were available ad libitum. Individual animal intake was recorded each day via the Grow Safe System (Grow Safe Systems Ltd., Airdrie, AB).

Animals were weighed full and empty on two consecutive days prior to the trial's start date and again immediately following the trial's completion date. During the trial, full weights were measured during wks 1, 2, 4, 5, 6, 7, 9, 11, 13 and 15.

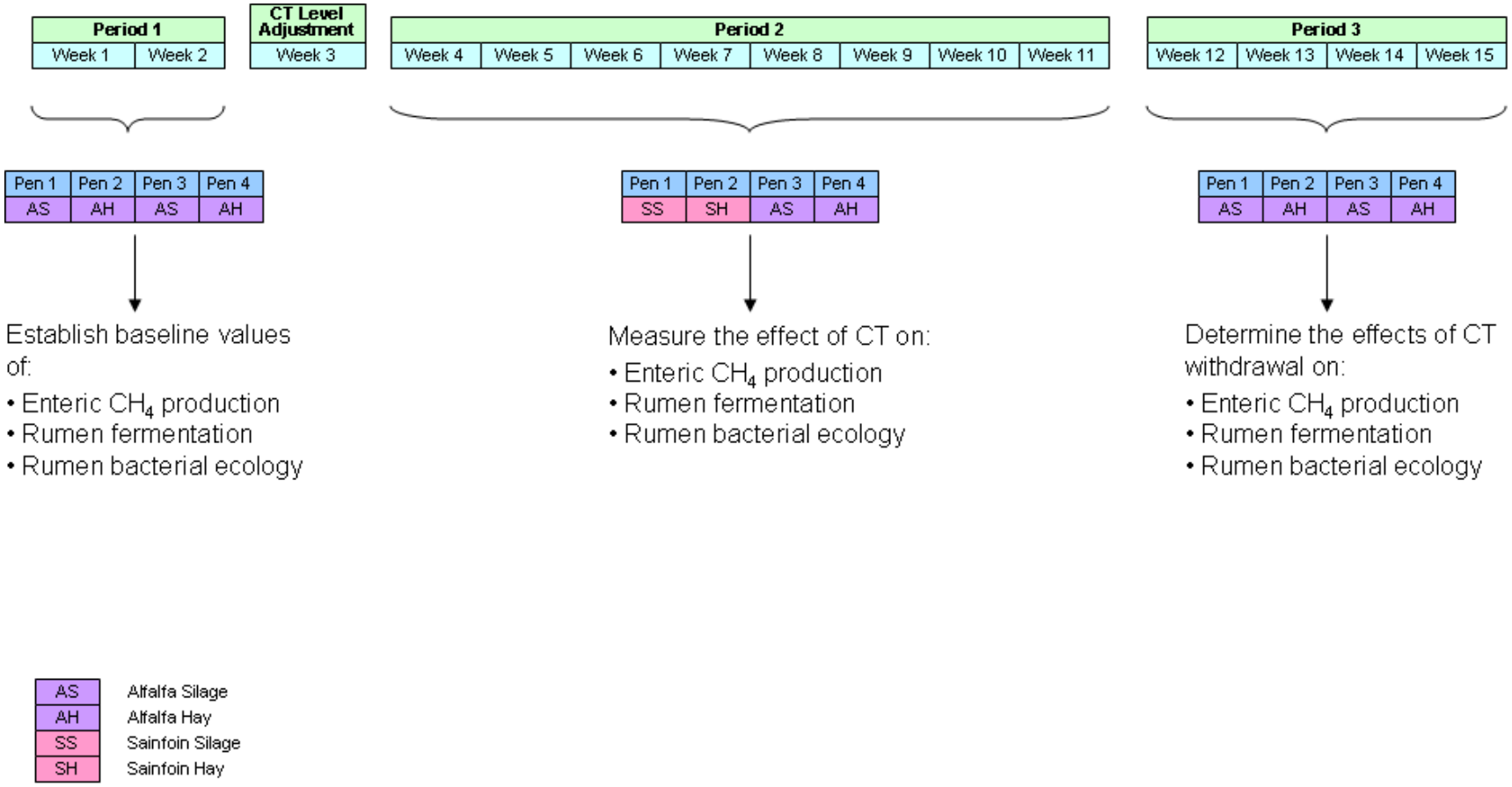
All steers received a 2 mL vitamin ADE injection two wks prior to the onset of the trial and again toward the end of the trial. Canadian Council on Animal Care guidelines were followed in the management and care of all animals (CCAC, 1993).

4.3.3 Feed Sampling and Analysis

Feed samples were collected daily and composited by wk. A portion of the composited samples were dried in a forced air oven at 60˚C for no less than 48 hours to determine DM content. A second portion of composited samples were freeze dried using a Genesis 25LE (VIRTIS, Gardiner, NY) freeze dryer. After drying, both sets of feed samples were ground using a Cyclotec tecater 1093 Sample Mill (Foss Analytical, Denmark) fitted with a 1-mm screen.

Oven dried samples were analyzed for crude protein (CP) using a Leco NS 2000 (Leco Corporation, St. Joseph, MI) and ash (Method no. 942.05 Association of Official Analytical Chemists 1990). Gross energy was resolved using a Par 6300 Automatic Isoperibol Calorimeter (Moline, IL). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined with an ANKOM 200 fiber analyzer (Fairport, NY) as described by Komarek et al. (1993). In vitro organic matter digestibility (IVOMD) was determined by the method of Tilley and Terry (1963) using bovine inoculum. Mineral concentrations including phosphorous (P), potassium (K), and magnesium (Mg) were analyzed by flame atomic absorption spectroscopy (Method No. 968.08; AOAC 1990) using a Vista MPX CCD Simultaneous ICP-OES (Varian, Mississauga, ON).

Figure 4.1. Diagram of the feeding strategy used during the 15-week trial



Freeze dried samples were analyzed for CT via the butanol-HCl method (Porter et al. 1986). Extractable CT were extracted from each sample by adding a 20 mL solution of 7:3 (vol/vol) acetone/water containing 0.1% ascorbic acid and 10 mL diethyl ether [4.7:2.0:3.3 acetone:water:diethyl ether (vol/vol)] to 500 mg of freeze dried plant material. The mixture was centrifuged at 27,000 x *g* for 15 minutes (min) to precipitate solids. Samples were analyzed in duplicate and the supernatant from each extraction was combined and permitted to settle until the aqueous and organic phase were separated. The organic phase was removed and discarded. The aqueous phase, containing CT, was dried via rotary evaporation at 40˚C and centrifuged to remove debris. The supernatant was increased to a final volume of 100 mL with distilled water. Six mL of butanol/HCl solution [95% but-1-ol:5HCl (36%) (vol/vol)] were added to 1 mL of supernatant from each extracted sample as well as to the 1 mL of standard. Mixtures were incubated in a water bath at 100˚C for 75 min. Once color development occurred, the tubes were removed from the bath and cooled on ice. Absorbance of each tube was read at 550 nm with a spectrophotometer (Ultraspec Plus 4054, Pharmacia, Baie d'urfe', QC) using the butanol/HCl solution as a blank. A standard curve was developed (0, 0.25, 0.5, 0.75 and 1.0 mg CT mL^{-1}) with freshly prepared sainfoin CT standard solution (Berard et al. 2011). Absorbance measurements of alfalfa extract were subtracted from all sample absorbance measurements to account for any background color development caused by compounds, other than CT, naturally present in plant material.

4.3.4 Enteric Methane Emissions and Analyses

Enteric CH_4 emissions were measured in each yearling steer at the beginning of weeks 1, 2, 4, 5, 6, 7, 9, 11, 13 and 15 using the SF_6 technique (Boadi et al. 2002). Stainless steel permeation tubes containing SF_6 were placed into the rumen of each steer

using a speculum 18 days prior to the start of the trial. Each permeation tube released SF_6 at a known rate, ranging from 338 to 568 ng SF_6 min^{-1}. Twenty-four h gas sample collection was accomplished by capturing exhaled gas from the nose and mouth of each steer using a halter fitted with capillary tubing connected to a pre-evacuated (40 mm Hg) stainless steel collection canister. Gas collection apparatuses were also hung on the north and south side of each pen to collect background air samples. These samples were later used to correct expired gas concentrations. The mean ambient temperatures during the CH_4 gas sampling period ranged between -33.3 and 10.8˚C, respectively (Figure 4.2.). Collected canisters were pressure checked to ensure pressures were less than 650 mm Hg and greater than 200 mm Hg. Values beyond this range were indicative of an incomplete gas collection and a second 24-h sample collection was performed immediately. Canisters were then pressurized with 110 kpa of nitrogen (N_2) to prevent contamination prior to analysis and to facilitate injection of the samples into the sample loop of a gas chromatograph (CP-3800, Varian, Mississauga, ON). Sulphur hexafluoride present in the sample was measured using an electron capture detector, whereas CH_4 was quantified using a flame ionization detector (Boadi et al., 2002). Prior to sample injection, the gas chromatograph was calibrated with prepared standards for SF_6 (20.73 ppt SF_6 – Scott Marrin Inc., Riverside, CA) and CH_4 (100.1 ppm CH_4 – Supelco, Mississauga, ON). The concentration of SF_6 and CH_4 was then determined from the peak area and retention time of the gas sample. Enteric CH_4 production from individual steers was determined using corrected SF_6 and CH_4 concentrations and the following equation:

$$CH_4 \text{ (L min}^{-1}\text{)} = \text{Permeation tube } SF_6 \text{ release rate (L min}^{-1}\text{)} \times [CH_4] / [SF_6]$$

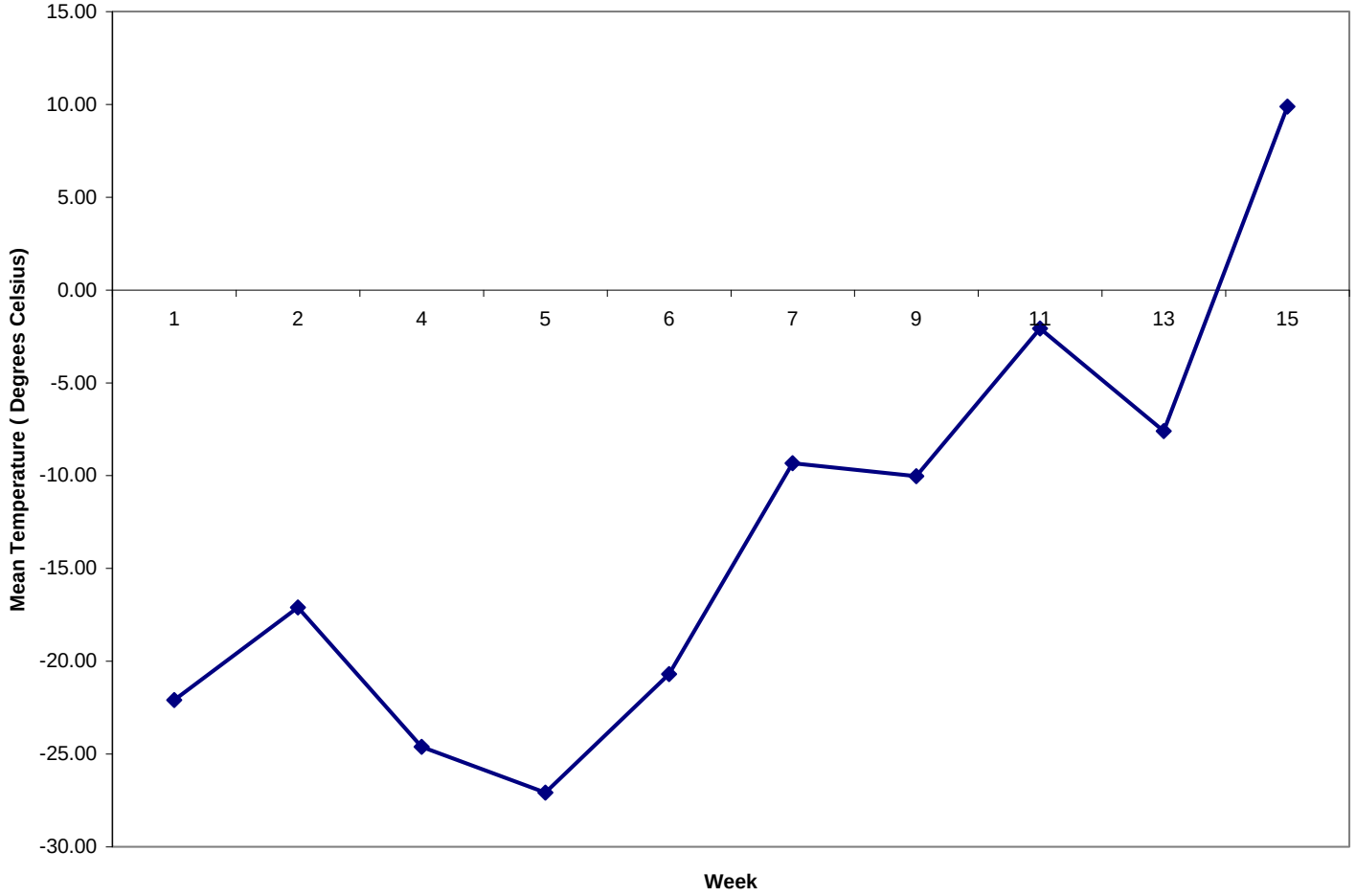


Figure 4.2. Mean ambient temperature (˚C) during the 15-week trial

4.3.5 Rumen Fluid Sample Collection and Preparation

Rumen fluid was collected from steers immediately before they were fitted with a halter and canister for CH_4 collection. Roughly 300 mL of rumen fluid was acquired from each steer at the beginning of weeks 1, 2, 4, 5, 6, 7, 9, 11, 13 and 15 using a Geishauser oral probe (Geishauser 1993). The initial 100 mL of fluid was discarded to avoid contamination from saliva. Following collection, 15 mL of rumen fluid was centrifuged for 55 min at a speed of 2,390 x *g*. Two portions of 4 mL supernatant were each transferred to 15 mL centrifuge tubes containing 0.8 mL of metaphosphoric acid and frozen for subsequent VFA analysis. The pellets from each sample were frozen in 0.9% saline for microbial community analysis.

4.3.6 VFA Analysis

Volatile fatty acid concentration was determined according to the method described by Erwin et al. (1961). Frozen samples were thawed at room temperature and mixed with 0.4 ml of 25% sodium hydroxide and 0.64 ml of 0.3M oxalic acid. Samples were then centrifuged at 1,860 x *g* for 20 min. Approximately 1.5 ml of supernatant was drawn from each sample, deposited into individual GC vials and tightly capped. Volatile fatty acid concentrations were determined using a Clarus 500 gas chromatograph (PerkinElmer Canada Inc., Woodbridge, ON) fitted with an auto-sampler (PerkinElmer Canada Inc., Woodbridge, ON). Both the injector and detector temperatures were set at 200˚C. The initial column temperature began as 175˚C and rose to 200˚C. The run-time was 21.25 min followed by a thermal stabilization period of approximately two min.

4.3.7 Microbial Community Analysis

As described above, microbial community analysis of rumen fluid was conducted using the frozen rumen fluid pellets. Pellets from the same three randomly selected

animals per dietary treatment were analyzed during weeks 2, 4, 7, 9 and 15. Total DNA was extracted using a ZR Fecal DNA Kit (D6010, Zymo Research Corp., Orange, CA) according to the manufacturer's instructions.

4.3.8 Terminal-Restriction Fragment Length Polymorphism

Polymerase chain reaction (PCR) amplification of the archaeal 16S rRNA gene was performed on each of the extracted samples using Arc 109F (5'-ACKGCTCAGTAACACGT-3') and Arc 934R (5'-GTGCTCCCCCGCCAATTCCT-3') primers (Microbial Community Analysis III, 2007). Only the five-prime end of the forward primer was tagged with a fluorescent molecule. Amplification was performed using a TC-512 (Techne Inc., Burlington, NJ) thermocycler and the following program: initial denaturation of five min at 94˚C, followed by 35 cycles of one min denaturation at 94˚C; 30 seconds (sec) of primer annealing at 51.6˚C; two min for extension at 72˚C and a final extension of 10 min at 72˚C. The correct size and quantity of the PCR products was verified by agarose gel electrophoresis before T-RFLP analysis.

Terminal restriction fragments (TRFs) were then produced by digesting PCR products with the restriction enzyme M*se*1(T^TAA) (New England Bio Labs, Ipswich, MA). A mixture of 16.6 µl of PCR product, 1 µl of restriction enzyme, 0.2 µl of 10 mg mL^{-1} bovine serum albumen and 2 µl of 10x reaction buffer was made for each sample and incubated at 37˚C for 5 h.

Following digestion, restricted samples were desalted using a mixture of 0.25 µl 2mg mL^{-1} glycogen and 2.0 µl of 3M NaOAC (pH 5.2). To the mixture, 56 µl of iced 95% vol/vol ethanol was added and centrifuged at 21,000 x *g* for 15 min at 4˚C. The pellet was rinsed twice with 200 µl of iced 70% vol/vol ethanol. Samples were

centrifuged for 5 min between each rinse. Samples were air dried and re-suspended in 15 µl of sample loading solution (SLS) (Beckman Coulter Inc., Fullerton, Ca).

The size of the resulting TRFs in each sample was determined using a CEQ 8800 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA). Three µl of digested sample, 28.5 µl of SLS and 0.75 µl of 1000 base-pair DNA size standard (Beckman Coulter Inc., Fullerton, CA) were mixed and applied to the CEQ's capillaries in triplicate. The CEQ software (Version 9.0; Beckman Coulter Inc., Fullerton, CA) was used to analyze the data. To eliminate unwanted background noise, a minimum level of fluorescence was defined in the machine. The background was determined by using a negative control PCR product. Fragment data output came in two forms, an electropherogram which is a series of colored peaks representing the microbial community, and a numerical table that indicates the size and height of each peak. The height of each peak represents the proportion of each population in the community relative to each other. Samples that did not appear to run properly, based on the above outputs, were deleted and repeats were performed.

4.3.9 Clustan Analysis

Binary TRF data were grouped according to dietary treatment and sample wk. ClustanGraphics 7 (Edinburgh, Scotland) software was employed to facilitate hierarchical clustering of the binary data. Hierarchical clusters indicate the similarity of microbial profiles by grouping samples together based on similarity. Average cluster linkage analysis was based on Jaccard's similarity coefficient.

4.3.10 Detrended Canonical Correspondence Analysis

To investigate the main factors affecting methanogen community profiles, an ordination biplot was constructed using Canoco 4.5 software (2002). Methanogen

profiles resulting from each dietary treatment in wks 2, 4, 7, 9 and 15 were analyzed against the following environmental variables: average weekly temperature (˚C), intake as a percent of body weight (%BW), CP intake kg d^{-1}, gross energy intake MJ d^{-1}, CT intake g d^{-1} and CH_4 emissions as a percent of gross energy intake (%GEI).

4.3.11 RT-PCR Analysis

Real time-polymerase chain reaction analysis was conducted to examine the microbial community in the rumen. Analysis was carried out by pooling 15 µl of extracted DNA from 60 rumen fluid samples based on dietary treatment and experimental wk into 20 samples. Deoxyribonucleic acid concentration of the pooled samples was measured at a wavelength of 260nm using a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter Inc., Fullerton, CA). Each pooled sample was diluted 10 times to achieve a working concentration of approximately 10 ng μl^{-1} of DNA. A standard curve was also prepared by combining equal volumes of one sample from every dietary treatment within each experimental week. The DNA concentration of the standard curve sample was also measured spectrophotometrically and diluted 10 times to achieve a working concentration of roughly10 ng μl^{-1}. The standard curve was then produced via a 7-fold serial dilution with an end concentration of 0.078 ng μl^{-1}.

The primers used for RT-PCR are listed in Table 4.1. All primers were previously assembled and tested by Khafipor et al. (2008) except for the methanogenic primers which were taken from the literature (Ohene-Adjei et al. 2004). Primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB).

Real time- PCR was accomplished using an AB 7300 system (Applied Biosystems, Foster City, CA) and sequence detection software (Version 1.3; Applied Biosystems, Foster City, CA). Each reaction had a final volume of 25 µl and was run in

Table 4.1. Primers used in real time polymerase chain reaction (RT-PCR) analysis of rumen fluid samples from steers fed sainfoin or alfalfa diets

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.
	518-534R	ATTACCGCGGCTGCTGG	56.2	64.7		1993
Ciliate Protozoa	UPorCil1F	GCTTTCGWTGGTAGTGTATT	50.2	20.0	234	Sylvester et al.
	UPorCil1R	CTTGCCCTCYAATCGTWCT	50.4	47.4		2004
Methanogenic Archaea	MB1174F	GAGGAAGGAGTGGACGACGGTA	60.6	59.1	232	Ohene-Adjei et al.
	Arch 1406-1389R	ACGGGCGGTGTGTGCAAG	60.0	66.7		2007
Streptococcus bovis	SBovis1F	TTCCTAGAGATAGGAAGTTTCTTCGG	57.9	42.3	127	Stevenson and Weimer
	SBovis1R	ATGATGGCAACTAACAATAGGGGT	57.9	41.7		2007
Butyrivibrio fibrisolvens	ButFib2F	ACCGCATAAGCGCACGGA	58.8	61.1	65	Stevenson and Weimer
	ButFib2R	CGGGTCCATCTTGTACCGATAAAT	55.7	45.8		2007
Prevotella ruminicola	PreRum92862F	GCGAAAGTCGGATTAATGCTCTATG	58.5	44.0	78	Khafipoor et al.
	PreRum92862R	CCCATCCTATAGCGGTAAACCTTTG	59.3	48.0		2008
Ruminicoccus flavefaciens	RumFla1F	CGAACGGAGATAATTTGAGTTTACTTAGG	57.5	34.5	132	Denman and McSweeney
	RumFla1R	CGGTCTCTGTATGTTATGAGGTATTACC	59.3	42.9		2006
Ruminicoccus albus	RumAlb1F	CCCTAAAAGCAGTCTTAGTTCG	54.3	45.5	176	Wang et al.
	RumAlb1R	CCTCCTTGCGGTTAGAACA	53.8	52.6		1997
Lactobacillus spp.[1]	Ulac16S1F	AGCAGTAGGGAATCTTCCA	51.5	47.4	345	Walter et al. 2001
	Ulac16S1R	ATTCCACCGCTACACATG	51.1	50.0		Lan et al. 2004

[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.

triplicate in optical reaction plates (Applied Biosystems, Foster City, CA) sealed with adhesive film (Applied Biosystems, Foster City, CA). Individual reactions contained 5 µl of water, 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1.25 µl of forward primer, 1.25 µl of reverse primer and 5 µl of sample DNA. The program for each set of primers was slightly unique. The program for the methanogenic archaea primers consisted of an initial denaturation of 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec with an annealing/extension step of 63˚C for 30 sec/ 72˚C for 30 sec. The same program was used for the ciliate protozoa primers however the annealing/extension step was altered to 54˚C for 30 sec/ 72˚C for 1 min. For the bacterial primers, each program consisted of an initial denaturation of 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 1 min.

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

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

Relative quantification was accomplished using the standard curve method outlined in Applied Biosystem's User Bulletin #2 (1997). All samples were normalized against Eubacteria results and period 2 was designated as the calibration period.

4.3.12 Statistical Analysis

Statistical analysis was conducted using a repeated measures model. A spatial power covariance structure was applied to the CH_4 and VFA data sets because time interval between sample measurements was unequally spaced. For all other data sets,

covariance structure was selected based on best fit statistics which was typically compound symmetry. Exceptions to this include NDF, P and K content of diets in addition to average daily gain (ADG) which all utilized a heterogeneous compound symmetry structure. All statistical analyses were employed with PROC MIXED (SAS Inst., Cary, NC), with least square means (LSMEANS) and resulting standard errors reported. It is important to note that data from weeks 2, 4, 5, and 6 were not included in the CH_4 analysis. Although measurements were made, only a limited number of data points were available as a consequence of equipment failure associated with inclement weather. Further, CH_4 emission data from collection canisters that did not meet final pressure designations, indicating incomplete collection, were also omitted from statistical analysis. The numbers of observations are reported in all cases and the following model was used:

$$Y_{ijk} = \mu + T_i + A_{ij} + W_k + (TW)_{ik} + \epsilon_{ijk}$$

Where Y_{ijk} = trait under consideration; μ = overall mean; T_i = dietary treatment (i = 1,2,3,4); A_{ij} = animal within treatments; W_k = week (k = 1…15); $(TW)_{ik}$ = treatment x week interaction; ϵ_{ijk} = experimental error term. Means were separated at the 5% level of significance using Fisher's Protected LSD test for all data sets.

Multivariate analysis was employed by Canoco 4.5 software (2002) to distinguish the effect of environmental parameters on the methanogen profiles of rumen fluid within treatments.

A two-tailed t-test was used to depict significance between hay and silage diets for all resulting RT-PCR data. Means were separated at a 5% level of significance. Standard errors reported within graphs were determined according to the standard curve method outlined in Applied Biosystem's User Bulletin #2 (1997).

4.4 RESULTS

4.4.1 Diet

The chemical composition (DM basis) of diets received by steers over the course of the trial is illustrated in Table 4.2. In period one, DM % of the AS diets was lower than AH. Gross energy (GE), CP, NDF, ADF and IVOMD were similar for silage and hay. Condensed tannin content was 0.0 mg g^{-1} and 1.8 mg g^{-1} on a DM basis in AH and AS respectively.

In period two, the DM % of both SH and AH was similar. However, the DM% of SS and AS was significantly different with values of 38.5% and 47.4% respectively. Sainfoin silage contained the greatest quantity of CP among the diets (18.3% DM basis) which was significantly greater than SH (12.4% DM basis) and AS (15.5% DM basis). Crude protein concentrations did not differ between SS and AH. Sainfoin silage also possessed the greatest GE content (4.4 Kcal g^{-1} DM basis) where as all other diets were similar. Neutral detergent fiber content was similar for all diets. Sainfoin silage and AS had comparable ADF values, however SH possessed significantly more ADF than AH. In-vitro digestibility of sainfoin was similar and significantly lower than that of the alfalfa. This is likely due to the presence of tannin in the SS (11.9 mg g^{-1} DM basis) and SH (10.5 mg g^{-1} DM basis) versus nearly non-existing levels of tannin in AS (1.7 mg g^{-1} DM basis) and AH (1.1 mg g^{-1} DM basis).

In period three, the DM content of the AS and AH diets differed with AS having higher moisture. Gross energy, CP, NDF, ADF and IVOMD were similar for both feeds. Although tannin content was very low in both the AH (0.4 mg g^{-1} DM basis) and AS (1.1 mg g^{-1} DM basis), these values were significantly different.

Table 4.2. Chemical composition (DM basis) of sainfoin silage (SS), sainfoin hay (SH), alfalfa silage (AS) and alfalfa hay (AH) fed to steers.

	Period 1			Period 2					Period 3		
Chemical Composition	AS	AH	SE	SS	SH	AS	AH	SE	AS	AH	SE
DM (%)	49.3 [a]	81.1 [b]	2.18	38.5 [a]	81.4 [b]	47.4 [c]	83.6 [b]	1.26	49.6 [a]	80.4 [b]	2.18
GE (Kcal g^{-1})	4.3	4.3	0.03	4.4 [a]	4.3 [b]	4.3 [b]	4.3 [b]	0.02	4.3	4.3	0.03
CP (%)	15.1	16.7	0.80	18.3 [a]	12.4 [b]	15.5 [c]	17.2 [ac]	0.46	16.2	16.9	0.80
NDF (%)	51.8	52.6	0.42	49.3	51.7	50.9	47.4	2.36	51.8	48.1	2.42
ADF (%)	36.1	39.4	2.00	38.7 [a]	41.1 [ac]	35.8 [ab]	35.3 [b]	1.15	36.6	34.2	2.00
P (%)	0.26 [a]	0.20 [b]	0.011	0.31 [a]	0.28 [ab]	0.24 [b]	0.16 [c]	0.024	0.28 [a]	0.17 [b]	0.011
K (%)	2.49	2.73	0.116	2.01	2.05	3.42	2.52	0.205	2.82 [a]	2.31 [b]	0.122
Mg (%)	0.36	0.42	0.054	0.40	0.31	0.31	0.37	0.031	0.40	0.35	0.054
Digestibility (%)	64.6	65.2	1.91	61.5 [a]	60.2 [a]	65.0 [b]	65.6 [b]	1.02	64.2	61.2	1.91
Tannin (mg g^{-1})	1.8 [a]	0.0 [b]	0.26	11.9 [a]	10.5 [a]	1.7 [b]	1.1 [b]	0.86	1.1 [a]	0.4 [b]	0.08
Pens receiving diet (n)	2	2		1	1	1	1		2	2	

[a,b] Within a row, means with different superscripts differ within period.

4.4.2 Animal Performance

The effect of diet on animal intake and performance for the duration of the trial is illustrated in Table 4.3. In period one, steers receiving the AS diet experienced lower ($P < 0.05$) DM intake expressed as kg d^{-1} and %BW than animals fed AH. This resulted in GE intake (MJ d^{-1}) and CP intake (g d^{-1}) also being less ($P < 0.05$) for steers consuming silage. Conversely, CT intake was higher ($P < 0.05$) for steers fed AS than AH. It should be noted that the amount of CT consumed by steers fed AS was trivial (14.4 g d^{-1}) and only significant because CT was absent in AH. Average daily gain of animals was similar for both diets.

In period two, steers fed SS and AS had similar values for DM intake expressed as kg d^{-1} and %BW as well as similar levels of GE intake. They did differ ($P < 0.05$) in level of CP intake with sainfoin-fed steers eating slightly more protein (1.4 kg d^{-1}) than alfalfa-fed steers (1.2 kg d^{-1}) as a consequence of the higher CP content of the sainfoin diets. Conversely, steers fed SH had lower ($P < 0.05$) DM intake expressed as kg d^{-1} and %BW than steers fed AH, resulting in lower GE and CP intake. Condensed tannin intake (g d^{-1}) was similar for both sainfoin diets and higher ($P < 0.05$) than both alfalfa diets. Average daily gain was comparable for all diets.

In period 3, feed intake (kg d^{-1}, %BW) and GE did not differ in steers that continuously received AS and those that had previously received SH and SS. Interestingly, DM intake (kg d^{-1} and %BW) as well as CP intake and GE intake were consistently lower ($P < 0.05$) for these animals versus the steers that continuously received the AH diet. Level of CT intake was less than 10 g d^{-1} in all diets. Average daily gain was comparable for all steers.

Table 4.3. Effect of diet on animal intake and performance in steers fed sainfoin silage (SS), sainfoin hay (SH), alfalfa silage (AS) or alfalfa hay (AH).

	Period 1			Period 2					Period 3				
Parameter	AS	AH	SE	SS	SH	AS	AH	SE	AS	AH	AS	AH	SE
DM intake, kg d^{-1}	8.1 [a]	9.7 [b]	0.10	7.9 [a]	9.1 [b]	8.0 [a]	10.1 [c]	0.17	8.8 [a]	9.5 [a]	8.8 [a]	10.7 [b]	0.30
DM intake, %BW	2.7 [a]	3.1 [b]	0.06	2.5 [a]	2.8 [b]	2.5 [a]	3.0 [c]	0.05	2.4 [a]	2.6 [a]	2.5 [a]	2.8 [b]	0.08
GE intake, MJ d^{-1}	143.7 [a]	170.6 [b]	3.62	145.7 [a]	164.2 [b]	142.9 [a]	180.5 [c]	3.09	157.5 [a]	171.2 [a]	157.2 [a]	192.6 [b]	5.35
CP intake, kg d^{-1}	1.2 [a]	1.6 [b]	0.04	1.4 [a]	1.1 [b]	1.2 [c]	1.7 [d]	0.03	1.4 [a]	1.6 [b]	1.4 [a]	1.8 [c]	0.06
CT intake, g d^{-1}	14.4 [b]	0.0 [a]	0.41	96.3 [b]	96.1 [b]	14.5 [a]	11.4 [a]	2.89	9.4 [b]	3.8 [a]	9.3 [b]	4.3 [a]	0.21
ADG, kg d^{-1}	0.6	0.6	0.13	0.7	0.6	0.7	0.6	0.05	1.8	1.8	1.5	1.5	0.13
Pens receiving diet (n)	2	2		1	1	1	1		1	1	1	1	

[a,b] Within a row, means with different superscripts differ within period.

4.4.3 Methane

Enteric CH_4 emissions from steers throughout the course of the trial are provided in Table 4.4. In period one, no statistical differences ($P < 0.05$) in CH_4 production between diets could be detected regardless of method of expression.

In period two, differences in CH_4 production were detected between the SH and AH diets when expressed as L d^{-1} and L kg BW^{-1}. Sainfoin hay resulted in significantly less ($P < 0.05$) CH_4 formation than AH. However, once corrected for DMI and GEI, no differences in CH_4 production between the two diets were apparent.

In period three, significant differences in CH_4 production expressed as L d^{-1} and L kg BW^{-1} were observed between the pens of steers that had received SS in period two versus the steers that continuously received AS throughout the trial. The steers that were switched from the SS diet back to AS emitted more CH_4 than the other animals. When CH_4 production is corrected for GEI, all diets were similar except for the pen that had received SS in period two. This group of animals emitted more ($P < 0.05$) CH_4 expressed as a %GEI compared to all other steers.

4.4.4 Rumen Fermentation

Volatile fatty acid profiles of rumen fluid samples from steers in each dietary treatment are illustrated in Table 4.5. In period one, the quantity of total VFA (mmol L^{-1}) is similar for the AS and AH treatments which will be converted to sainfoin in period two. Rumen fluid from these steers also contains comparable concentrations of acetic acid, propionic acid, butyric acid and acetic:propionic acid ratio. The permanent AH and AS dietary treatment groups also have a similar quantity of total VFA and acetic acid which is significantly higher ($P < 0.05$) from the afore mentioned two treatments. The AH diet has the greatest concentration of propionic acid and accordingly the lowest

Table 4.4. Enteric CH_4 emissions expressed as L d^{-1}, L kg BW^{-1}, L kg DMI^{-1} and %GEI in steers fed sainfoin silage (SS), sainfoin hay (SH), alfalfa silage (AS) or alfalfa hay (AH).

	Period 1					Period 2					Period 3				
Methane	AS	AH	AS	AH	SE [1]	SS	SH	AS	AH	SE [1]	AS	AH	AS	AH	SE [1]
L d^{-1}	176.7	119.2	177.6	174.8	25.75	197.9 [ab]	181.3 [a]	199.9 [ab]	245.7 [b]	19.25	255.7 [b]	223.9 [ab]	179.7 [a]	208.4 [ab]	18.66
L kg BW^{-1}	0.61	0.42	0.61	0.58	0.075	0.60 [ab]	0.55 [a]	0.60 [ab]	0.72 [b]	0.056	0.70 [b]	0.61 [ab]	0.50 [a]	0.55 [a]	0.054
L kg DMI^{-1}	23.8	16.2	22.1	19.8	2.55	22.1	19.6	23.4	23.4	1.90	28.6 [b]	23.8 [ab]	21.1 [a]	19.6 [a]	1.85
% GEI	5.2	3.6	4.8	4.4	0.56	4.8	4.3	5.2	5.1	0.42	6.3 [b]	5.2 [a]	4.7 [a]	4.3 [a]	0.41

[a,b] Within a row, means with different superscripts differ within a period.

[1] SE for each dietary treatment is pooled within a period.

acetic:propionic ratio of all dietary treatments ($P < 0.05$).

In period two, rumen fluid samples from animals fed SS contained less ($P < 0.05$) total VFA, acetic acid, propionic acid, and butyric acid than samples from steers fed AS. However, samples from steers fed AS had a lower ($P < 0.05$) acetic:propionic ratio (4.5 mmol L^{-1}) than the samples from animals consuming SS (5.5 mmol L^{-1}). Rumen fluid from animals consuming SH diets contained less ($P < 0.05$) total VFA, butyric acid, propionic acid and acetic acid versus fluid samples from animals fed AH. Interestingly, the AH and SH diets did not differ in terms of their acetic:propionic ratios.

In period three, the AS diets contained similar quantities of total VFA, butyric and acetic acid. However, rumen fluid from the steers continuously-fed AS contained more propionic acid and subsequently a lower acetic:propionic ratio than rumen fluid from steers that had been fed SS in period two. No statistical differences could be distinguished between the VFA profiles of rumen fluid from the two groups of steers fed AH.

4.4.5 Clustan Analysis

Multivariate analysis, illustrated in Figure 4.3, indicates that cluster patterns did not change as a consequence of diet over time. Methanogens appear to be unaffected by inclusion of CT and method of forage preservation.

4.4.6 Discriminate Canonical Analysis

An ordination biplot illustrating the influence of temperature, intake (%BW), CP intake, GE intake, CT intake and CH_4 (%GEI) on rumen fluid profiles from test steers is demonstrated in Figure 4.4. Within the ordination biplot, parameter data is represented by arrows and the methanogenic profiles of rumen fluid are represented by an assortment of shapes. Distance between rumen fluid profiles indicates their dissimilarity. Distance

Table 4.5. Volatile fatty acid profiles of rumen fluid samples from steers fed sainfoin silage (SS), sainfoin hay (SH), alfalfa silage (AS) or alfalfa hay (AH).

VFA (mmol L^{-1})	Period 1				Period 2				Period 3				
	AS	AH	AS	AH	SS	SH	AS	AH	AS	AH	AS	AH	SE
Acetic acid	49.7 [a]	51.5 [a]	61.7 [b]	67.4 [b]	46.8 [a]	52.6 [a]	59.0 [b]	67.8 [c]	58.0 [a]	86.2 [b]	61.6 [a]	85.5 [b]	2.12
Propionic acid	9.9 [a]	11.0 [ab]	12.8 [b]	15.3 [c]	8.7 [a]	12.2 [b]	13.2 [b]	16.7 [c]	12.1 [a]	22.4 [c]	14.0 [b]	23.5 [c]	0.62
Acetic:Propionic	5.1 [b]	5.3 [b]	5.2 [b]	4.5 [a]	5.5[c]	4.4 [ab]	4.5 [b]	4.1 [a]	4.9 [c]	3.9 [a]	4.4 [b]	3.7 [a]	0.12
Butyric acid	4.7 [ab]	4.3 [a]	6.5 [c]	5.3 [b]	4.2 [a]	4.4 [a]	6.0 [b]	5.8 [b]	5.1 [a]	8.7 [b]	6.0 [a]	9.1 [b]	0.31
Total VFA	66.3 [a]	68.8 [a]	83.3 [b]	90.2 [b]	60.8 [a]	71.2 [b]	81.0 [c]	93.2 [d]	77.2 [a]	121.4 [b]	84.2 [a]	121.7 [b]	3.07

[a,b] Within a row, means with different superscripts differ within a period

between a rumen fluid profile and an arrow-head indicates the influence of the parameter on the rumen fluid profile. Arrows pointed in similar directions signify a positive correlation between parameters whereas arrows in opposite directions indicate a negative correlation. The length of an arrow illustrates the relative effects of the particular parameter it represents. The most obvious and significant effect is the negative correlation between CH_4 production and energy/DM intake. Archael populations also appear to change in their distribution according to diet type although this observation is not statistically strong ($P < 0.1$).

4.4.7 RT-PCR Data

The abundance of structural carbohydrate-fermenting bacteria within the rumen is demonstrated in Figure 4.5. Relative to values in period one, SS had a suppressive effect on all bacteria. This effect persisted into period three when the diet was switched back to AS. Conversely, a relative increase in the abundance of bacteria was observed when animals were fed SH in period two. This effect also continued into period three when the diet was changed back to AH. Relative to period one, the abundance of all bacteria except *Ruminococcus flavefaciens* declined in the rumen of animals fed AS in periods two and three. Alfalfa hay had the opposite effect of silage and stimulated growth of all bacteria in period two and period three except for *Butyrivibrio fibrisolvens* which declined in abundance in the latter period. Relative abundance of non-structural carbohydrate fermenting-bacteria is illustrated in Figure 4.6. During period two, SS and AS had a suppressive effect on *Lactobacillus species* but not *Prevotella ruminicola* or *Streptococcus bovis*. During period two, the relative abundance of all bacteria except *S. bovis* declined in the rumen fluid of steers fed the SH diet. *Lactobacillus spp.* declined in the rumen fluid profiles of steers fed AH during period

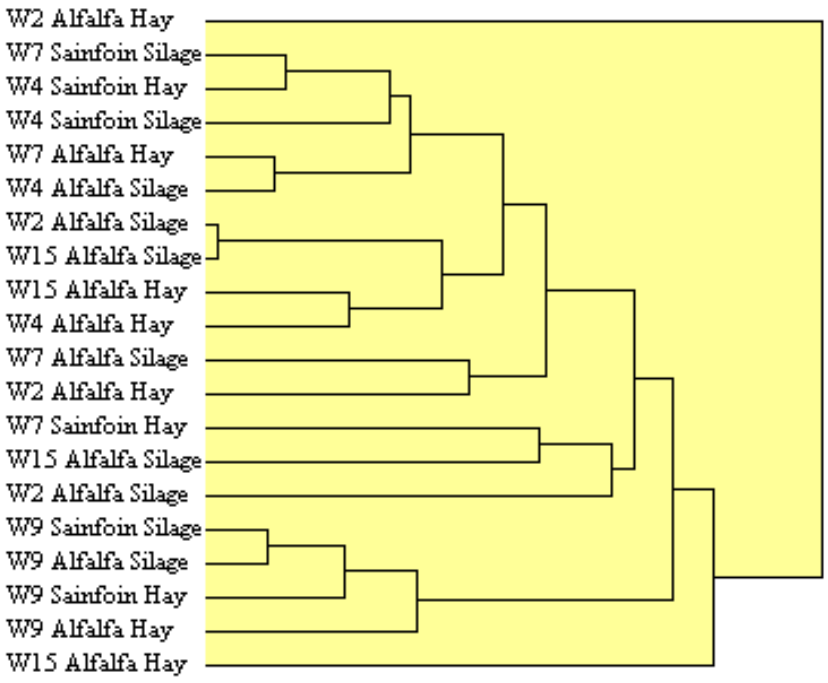


Figure 4.3. Cluster analysis of rumen methanogens grouped by diet. Average cluster linkage analysis is based on Jaccard's similarity coefficient.

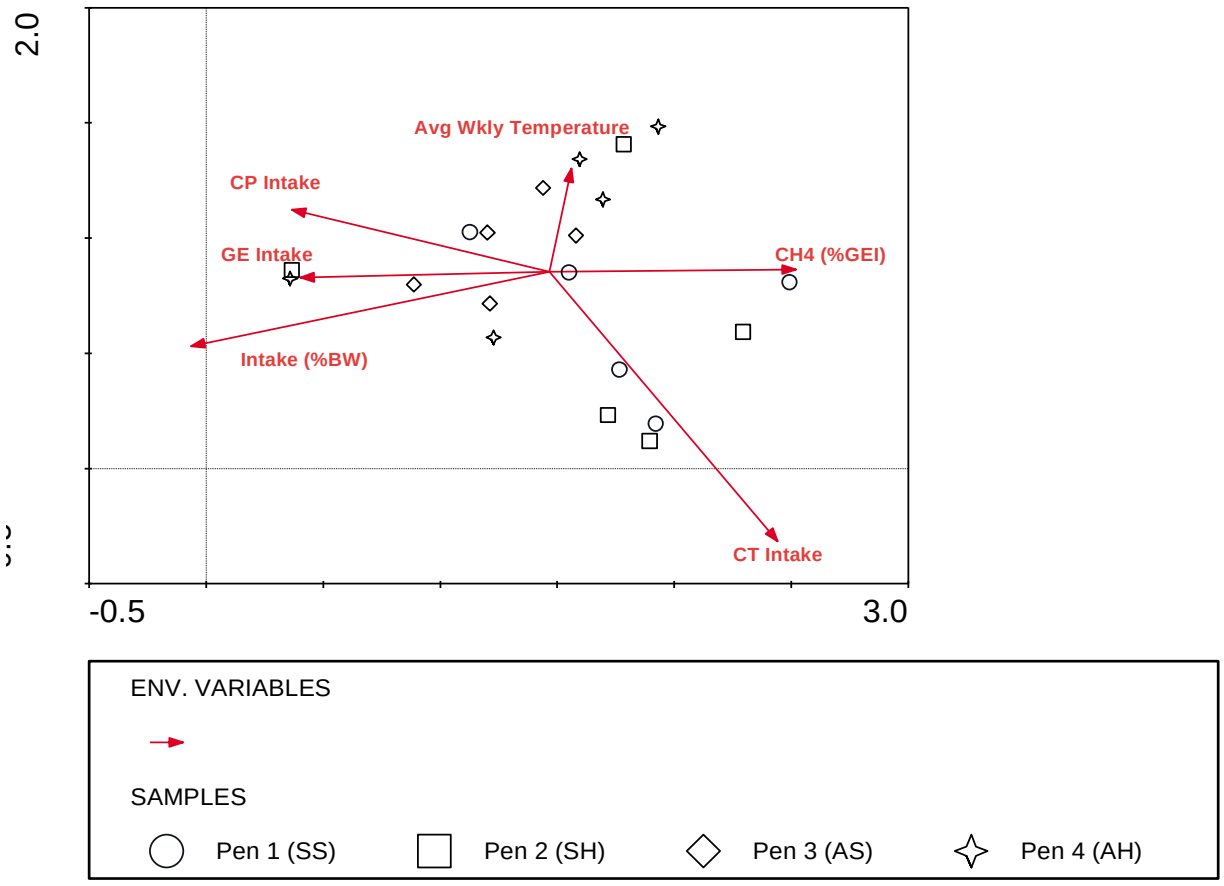


Figure 4.4. Ordination biplot illustrating the influence of temperature, intake as a percent of body weight, crude protein intake, gross energy intake, condensed tannin intake and methane production expressed as a percent of gross energy intake combined with diet type on the profile of methanogens in rumen fluid.

two but this effect was not seen in other bacteria.

Relative abundance of methanogens can be observed in Figure 4.7. Feeding SS had a suppressive effect on methanogens in period two, which carried over into period three when steers were switched back to an AS diet. Conversely, feeding SH stimulated the growth of methanogens relative to period one values. This same effect also continued into period three. The AH diet encouraged a ($P < 0.05$) greater relative abundance of methanogens in both periods two and three compared to the AS diet.

Relative abundance of ciliate protozoa from the rumen fluid of steers throughout the trial is indicated in Figure 4.8. Feeding sainfoin, whether in the form of silage or hay, appeared to increase the growth of protozoa in period two. This effect continued into period three, when sainfoin diets were replaced with alfalfa. Interestingly, animals fed the AS diet experienced a decline of protozoal abundance in period two, followed by an increase in period three. The exact opposite occurred in animals fed AH as populations increased quite drastically in period two and declined below base levels in period three.

4.5 DISCUSSION

Sainfoin possesses several agronomic characteristics that make it a sound candidate for use as a forage crop. For example, it has a deep tap root that allows the plant to be highly drought resistant and because it is a legume it has excellent nitrogen fixing capabilities (Koiuisto and Lane 2001). Additionally, sainfoin consumption has led to better growth rates in cattle as compared to alfalfa (Marten and Ehle, 1984). The higher level of animal performance observed when a diet contains low levels of CT has

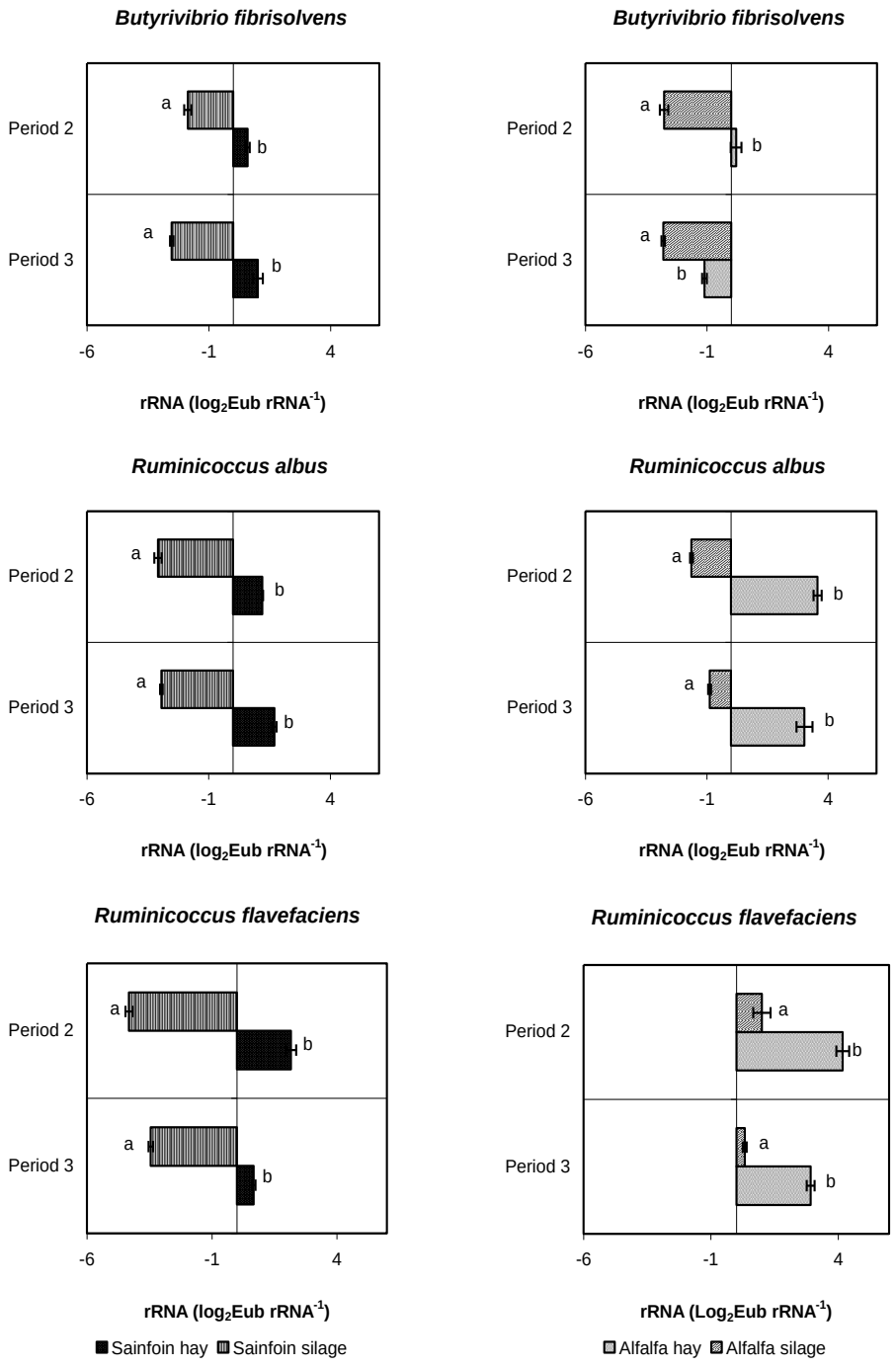


Figure 4.5. Relative abundance of structural carbohydrate fermenting bacteria in rumen fluid from steers fed sainfoin silage/hay and alfalfa silage/hay over 15-weeks. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to values in period one. Different letters denote significant differences within period.

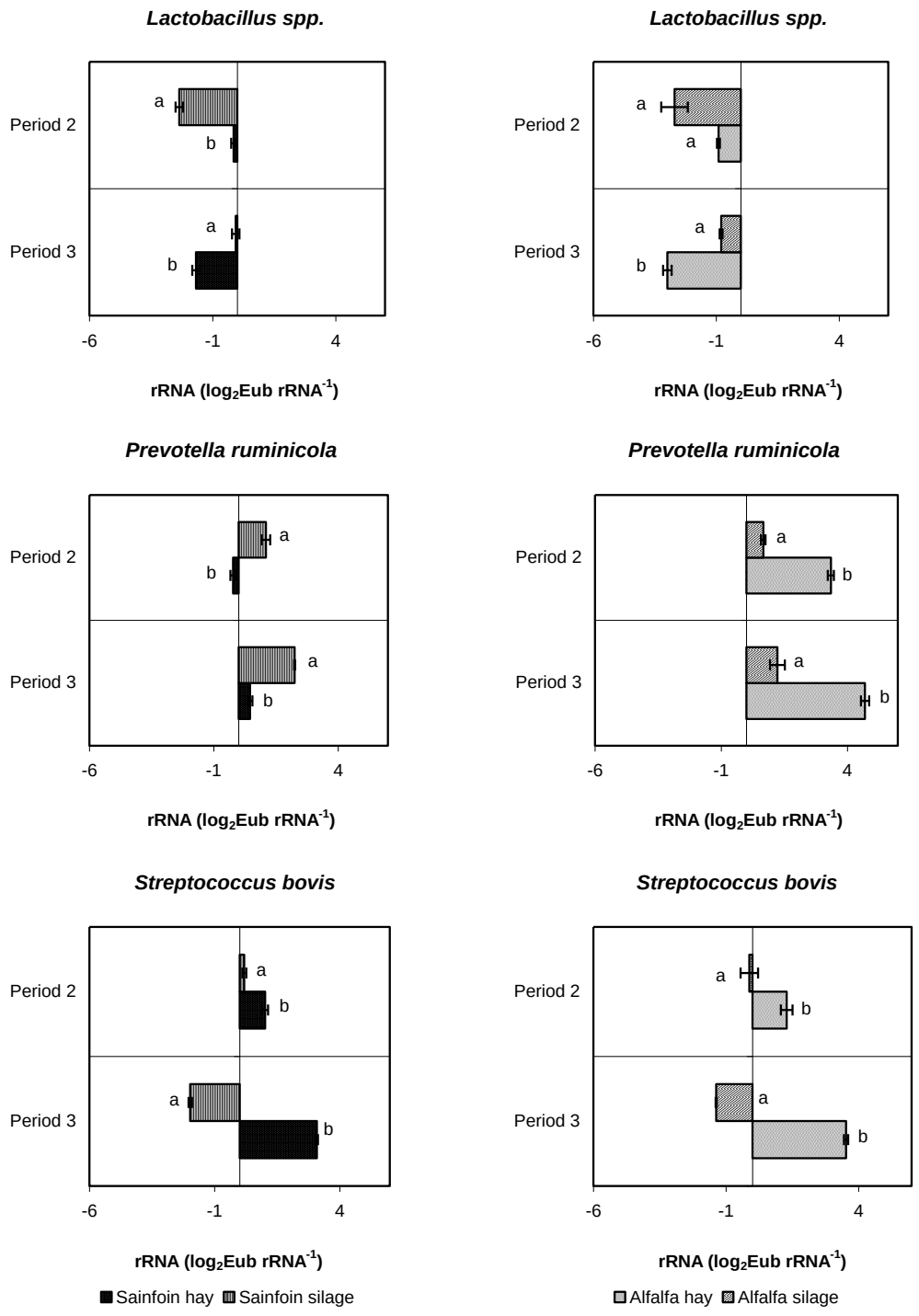
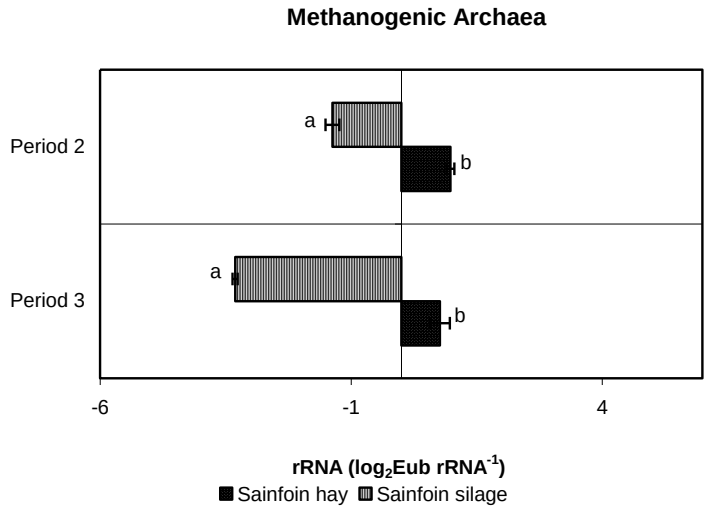


Figure 4.6. Relative abundance of non-structural carbohydrate fermenting bacteria in rumen fluid from steers fed sainfoin silage/hay and alfalfa silage/hay diets over 15-weeks. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to values in period one. Different letters denote significant differences within period.



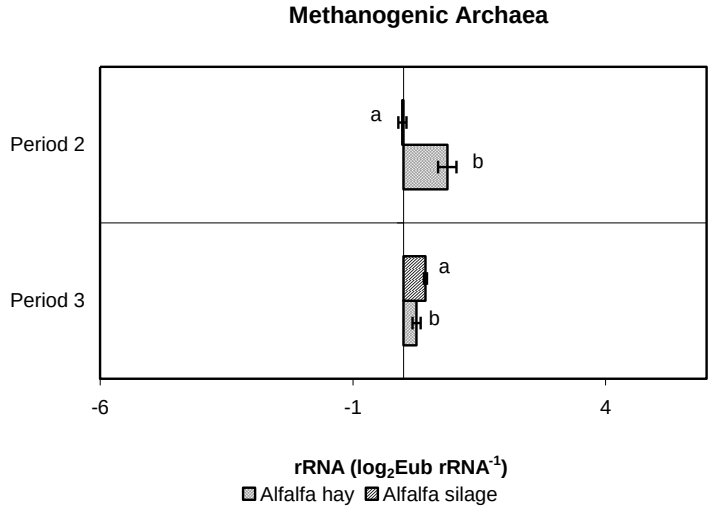
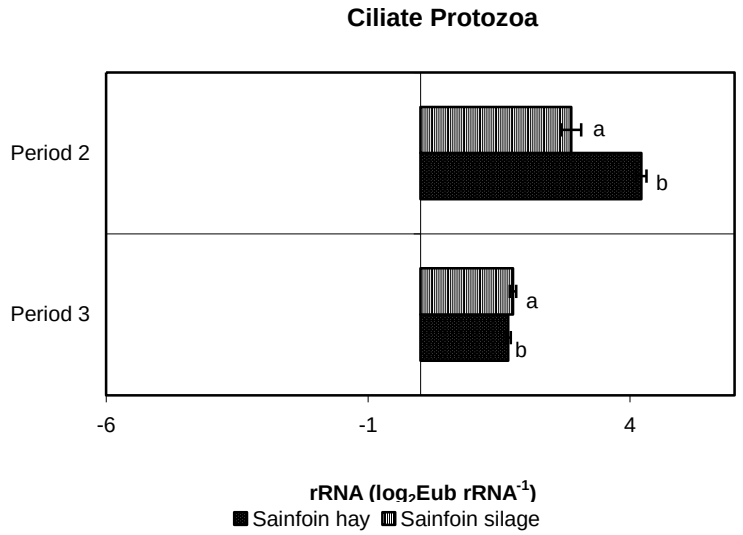


Figure 4.7. Relative abundance of methanogenic archaea in rumen fluid from steers fed sainfoin silage/hay and alfalfa silage/hay diets over 15-weeks. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to values in period one. Different letters denote significant differences within period.



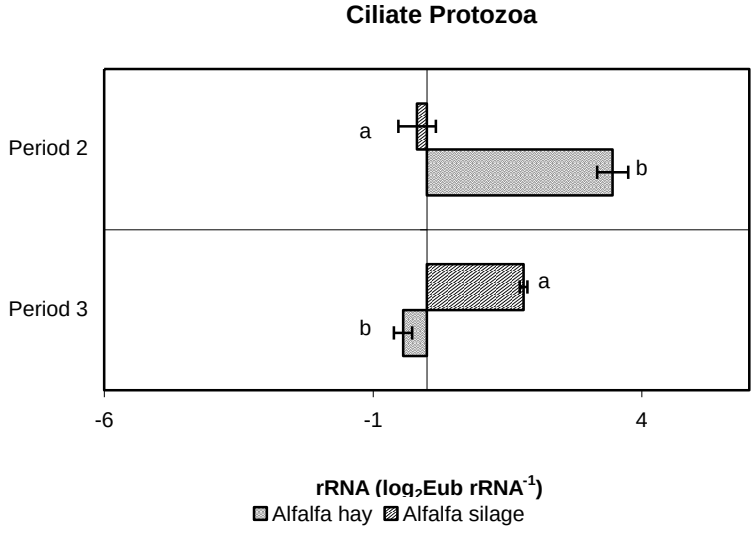


Figure 4.8. Relative abundance of ciliate protozoa in rumen fluid from steers fed sainfoin silage/hay and alfalfa silage/hay diets over 15-wks. Abundances are log-2 transformed values expressed as copies of specific rRNA per copy of eubacterial rRNA. All abundances are expressed relative to values in period one. Different letters denote significant differences within period.

been attributed to the protection of feed protein from degradation in the rumen, resulting in an increase in the flux of essential amino acids to the small intestine and an increase in the absorption of essential amino acids to the blood (Waghorn and Shelton 1997). In spite of these properties, sainfoin is seldom used for forage production in Western Canada. This, in part, may be due to difficulties associated with plant palatability. It has been demonstrated that cattle consuming CT-containing forages may experience suppressed DM intake due to the astringent nature of tannins (Goel et al. 2005). The sainfoin diets in our research contained approximately 1% CT on a DM basis (Table 4.2) and did not appear to affect palatability as steers fed SS and SH experienced DM intakes of 2.5 and 2.8 %BW (Table 4.3) respectively. Sainfoin grown in a Manitoba production environment and preserved as round bale silage/hay effectively supported an acceptable growth rate for backgrounding steers during a western Canadian winter. Backgrounding rations are designed to achieve a predetermined daily rate of gain. It is reasonable to expect medium framed steers to have gains of between 0.68 and 0.91 kg d^{-1}. Crude protein concentrations of both sainfoin diets met National Research Council (1996) requirements for 660 kg steers gaining 0.91 kg d^{-1}. Steers receiving the SS and SH diets experienced gains of 0.7 and 0.6 kg d^{-1} respectively (Table 4.3) and did not differ significantly from those animals receiving alfalfa diets. It is possible that the cold temperatures experienced during periods one and two (Figure 4.2) suppressed steer weight gain. Exposure to extreme cold can have negative consequences on ADG in growing beef cattle as a result of heightened maintenance demands (Kennedy and Milligan 1978). Warmer ambient temperatures may be linked to the improved rates of gain observed in steers during period three.

Studies have reported that feeding CT-containing forages to ruminants reduces CH_4 emissions. Woodward et al. (2001) fed dairy cattle *Lotus* silage containing 2.6% CT versus ryegrass silage with no tannin. Milk production and DMI were significantly higher for cattle consuming *Lotus* but enteric CH_4 production was similar for both diets. However, when corrected for intake, the higher DMI of the *Lotus*-fed cattle led to significantly lower CH_4 emissions per kg DMI and per kg milk-solids produced. It should be noted that the *Lotus* fed by Woodward et al. (2001) had a significantly lower NDF content compared to the ryegrass diet. Since low fiber diets are typically associated with reduced CH_4 emissions (Johnson and Johnson 1995) their findings may also be influenced by differences in nutrient composition. In our research, NDF levels did not differ among diets (Table 4.2). In a subsequent experiment with grazing dairy cattle, Woodward et al. (2004) demonstrated that reductions in CH_4 are a direct result of feeding CT. When the effects of tannin were neutralized by polyethylene glycol (PEG) in cattle receiving a *Lotus* diet, CH_4 emissions were 13% higher than from cattle being fed the same diet without PEG.

In our research, a significant decline in enteric CH_4 production could only be observed in SH-fed animals compared to AH (Table 4.4) when expressed as L d^{-1} or L kg BW^{-1} but not as L kg DMI^{-1} or %GEI. It is possible that the sainfoin diets fed did not contain sufficient quantities of CT and as such enteric CH_4 emissions were not reduced to the same extent as that observed by Woodward et al. (2001). Beneficial effects of CT on ruminant animal performance, including reductions in enteric CH_4 formation, are typically experienced at dietary concentrations between 2-4% DM (Aerts et al. 1999). Our sainfoin diets only contained 1% CT on a DM basis. Additionally, CT from different plant sources vary in their ability to bind with proteins and carbohydrates (McAllister et al. 2005).

Condensed tannins with high molecular weights (MW) have been observed to precipitate less protein per unit weight than CT with low MW. The MW of CT from specific plant species is consistent and well documented. Sainfoin has been observed to contain high MW CT whereas *Lotus* species contain low MW CT (Barry 1989). Accordingly, CT originating from sainfoin may need to be fed at slightly higher concentrations than CT from *Lotus* species to achieve a similar impact on enteric CH_4 production from cattle.

Interestingly, the significant decline in CH_4 production (expressed as L d^{-1} and L kg BW^{-1}) observed when SH-fed animals are compared to AH-fed animals is absent when SS-fed animals are compared to AS-fed animals. Sainfoin silage and SH contained nearly identical concentrations of CT. Furthermore, digestibility which is positively correlated with CH_4 formation did not differ between the two diets (Table 4.2.). It is possible that a reduction in CH_4 was not observed when SS was compared to AS because animals consuming these diets experienced similar levels of DMI (Table 4.3.). It is generally accepted that an increase in feed intake lowers CH_4 losses as a percentage of daily energy intake (Blaxter and Clapperton 1965). The major effect of a higher feed intake is related to its impact on passage rate. Particles that pass quickly from the rumen experience less extensive fermentation since their exposure to rumen microorganisms is reduced. It should be noted that diets comprised entirely of roughage, comparable to those fed in our research, do not affect rate of passage to the same extent as concentrate or mixed diets (Mathison et al. 1998). Steers receiving the SH diet emitted less CH_4 than those receiving AH, however they also consumed less DM. Since rate of passage is not largely affected by level of intake when roughages are fed, it can be proposed that the amount of DM consumed by steers on the SH diet is responsible for the reduction in CH_4 production because there was less substrate available for fermentation in the rumen. This theory is

supported by the fact that the significant difference in CH_4 emissions between the SH and AH-fed steers disappears when CH_4 is expressed as L kg DMI^{-1} and as a %GEI. Another possible explanation for the lack of reduction in CH_4 production observed when SS and AS diets are compared, is the effect of ensiling on the chemical form of CT present in sainfoin and the consequent effect on fermentation in the rumen. Scharenberg et al. (2007) compared sainfoin that was fed as silage or hay to sheep. They found that although method of preservation did not alter total concentration of CT in either feed, it did significantly decrease the content of extractable tannin in SS. Extractable CT are those that are not bound to protein and fiber within the feed. It is likely that the ensiling process degrades the plant's structure leading to increased surface area and free molecules (plant protein and fiber) where CT can bind. Accordingly, Scharenberg et al. (2007) also observed a substantial increase in the amount of CT associated with the fiber and protein fractions in SS. Research by Makkar and Becker (1997) suggests that bound CT are inert and do not affect microbial fermentation as long as they remain bound. These findings are further supported by research conducted by Wang et al. (2006) who included PEG for specific inactivation of CT in the *in vitro* ruminal fermentation of silages containing sainfoin. The inclusion of PEG did not affect IVDMD, gas production or accumulation of VFA, microbial nitrogen or ammonia nitrogen inferring that the CT present in the ensiled forages had no major effects on ruminal fermentation. It is hypothesized that CT may affect rumen methanogenesis via two distinct mechanisms: an indirect effect caused by a reduction in fiber digestion leading to decreased H_2 production in the rumen or by directly inhibiting the growth of methanogens (Tavendale et al. 2005). If the CT in the SS diet remained bound, they would not have interacted with the prokaryotic or methanogenic populations in the rumen thus explaining the lack of a reductive effect on CH_4 formation.

Although we did not analyze the distribution of CT in our sainfoin diets, we observed a significant decline in the feed intake with the SS diet, as was observed by Scharenberg et al. (2007). Methane emissions expressed as L d^{-1} from animals fed SS and SH differed by only 8.4% and were not statistically significant but DMI of SS-fed animals was 13.2% ($P < 0.05$) lower than SH-fed animals.

Beauchemin et al. (2007) fed quebracho extract at three different dietary DM concentrations (0%, 1%, and 2%) to cattle consuming a barley silage-based diet for three, 28 day periods. They observed no effects on CH_4 emissions or digestibility. Interestingly, they did observe that increasing levels of CT linearly decreased total VFA and the proportion of acetate in the rumen. Accordingly, the acetate:propionate ratio was also reduced. The molar percentage of VFA's present in the rumen influence the production of CH_4. The presence of acetate and butyrate promote CH_4 production as both VFA's are part of H_2 producing reactions. Conversely, the reaction involved in propionate production requires H_2 and competes with methanogenesis (Moss et al. 2000). Thus, it is possible to decrease enteric CH_4 formation by increasing the proportion of propionate present in the rumen. Although reductions in CH_4 were not detected in the research conducted by Beauchemin et al. (2007), feeding CT appeared to stimulate favourable changes in ruminal fermentation. Such favourable changes in fermentation may be attributed in part, to the effect of CT on a specific group(s) of microorganisms. McSweeney et al. (1998), for example, observed a marked decrease in rumen cellulolytic bacteria including *R. albus* when CT from the legume shrub *Calliandra calothyrsus* was incorporated in the diet of sheep. *Ruminococcus albus* is known to produce acetate as a major fermentation end-product. Although Beauchemin et al. (2007) did not examine the direct effect of quebracho tannin on bacterial populations in their study, it is possible that

the effects they observed on fermentation result from an inhibitory effect of CT on specific rumen microorganisms. In our study, the CT containing sainfoin diets also resulted in less ($P < 0.05$) total VFA and acetate production. However, we did not observe a decrease in the acetate:propionate ratio of steers fed these diets because the presence of propionate in the rumen was also less. Benchaar et al. (2008) added cinnamaldehyde, a precursor of tannin, and quebracho CT to grass silage diets supplemented with corn and beet pulp. These diets were fed to lactating dairy cows but no significant effects of cinnamaldehyde or quebracho were detected on rumen fermentation or digestibility measures. Although the effects of CT on the molar proportions of VFA's present in the rumen have been inconsistent, there is evidence that CT can lead to fermentation conditions that lower ruminal CH_4 production.

In period three of the present study, hay diets resulted in significantly greater total VFA, acetic acid and propionic acid production than silage diets. Hay diets also resulted in significantly lower acetic:propionic acid ratios (Table 4.5). These findings indicate that hay diets generated more favorable rumen fermentation profiles for reducing enteric CH_4 formation than silage diets. This contradicts speculations by Sundstøl (1981) which state that ensiling, rather than drying forages, will most effectively decrease CH_4 production as a %GEI due to decreased digestion in the rumen.

The microbial community inhabiting the rumen of cattle is incredibly diverse. Within the rumen, a specialized group of microbes, the methanogenic archaea, are ultimately responsible for CH_4 production. Additionally, several other microorganisms have a significant influence on CH_4 formation either by promoting an environment suitable for methanogen survival or producing substrates used by the methanogens for growth (Morgavi et al. 2010). In our research, molecular techniques were employed to

observe the effect of feeding a CT-containing diet as well as method of forage preservation on specific rumen bacteria involved in enteric CH_4 production. Using cluster analysis we observed no changes in the rumen microbial community structure of archaea in any of the experimental animals. This is surprising as CT was fed for nearly three months. Research conducted by Tavendale et al. (2005) demonstrated that a polymeric CT fraction from *Lotus pedunculatus* completely inhibited CH_4 production and growth of a rumen methanogen in broth culture. Based on these findings, we anticipated feeding a CT-containing diet would either mitigate or alter the composition of the methanogen populations inhabiting the rumen followed by a possible recovery as they became adapted to the CT. It is likely that the effect of CT-containing fractions on pure cultures of methanogens is greater than CT-containing plants in mixed rumen fluid due to the lack of an additional target protein for CT binding. It is also possible that the concentration of CT in our sainfoin diets was insufficient and therefore did not elicit a response on the rumen archael community. Nevertheless, our cluster data suggests that the archeal population of the rumen is stable and diminutively affected by diet, and or ambient temperature. Our findings are in agreement with research conducted by Hook et al. (2009) and Guo et al. (2008) who observed the effects of specific feed additives possessing antimicrobial properties on methanogenic populations. Hook et al. (2009) fed lactating dairy cattle a high starch diet containing various concentrations of the antimicrobial ionophore, monensin. They were unable to observe any significant changes in the archael population. Guo et al. (2008) supplemented *in vitro* cultures of methanogens with tea saponins. Although they did detect a decline in CH_4 production, the effect was not related to a change in methanogen populations but rather to changes in fungal and protozoal populations.

Discriminate canonical analysis of our archael T-RFLP data in conjunction with animal performance variables and quantified gross CH_4 emissions from test steers indicate significant changes in rumen function (Figure 4.4). The most pronounced effect was a negative correlation between CH_4 and GEI. This is expected as it has been demonstrated that as DMI increases, %GEI lost as CH_4 decreases (Johnson and Johnson, 1995). There was also a significant effect ($P < 0.1$), although not strong, toward a change in the rumen fluid profiles of archael populations according to diet type. Thus we can conclude that, at best, in the present study the effects of CT on archaea were subtle.

The use of RT-PCR allowed us to determine the relative abundance of specific rumen prokaryotes, methanogens and ciliate protozoa over the course of our experiment. We observed that microbial species varied significantly by period, forage type and method of forage preservation (Figure 4.5 and Figure 4.6). Research conducted elsewhere (Scalbert 1991) suggests that tannins are toxic to microorganisms via three main mechanisms: enzyme inhibition, substrate and metal ion deprivation, and action on membranes. Jones et al. (1998) observed that CT extracted from sainfoin leaves inhibited growth and protease activity in *B. fibrisolvens* and *S. bovis*. As such, we hypothesized that rumen prokaryotic populations would be significantly affected by feeding CT-containing diets. Surprisingly, consumption of a CT-containing diet did not appear to have a major affect on the relative abundance of the examined rumen prokaryotes in our steers. Again, this absence of an affect may be because the concentration of CT in our diets was only 1%. The variable with the most notable affect on rumen prokaryotes in our research was method of forage preservation. Silage diets inhibited far more rumen microbial species than did hay diets. Plant structural carbohydrates, proteins and other organic polymers are degraded to their monomer components by primary anaerobic

fermenters. These monomers are then further converted into VFA's, CO_2 and H_2 by both the primary fermenters and other microbes that do not have the ability to hydrolyse complex polymers themselves (Morgavi et al. 2010). *Ruminicoccus albus* and *R. flavefaciens*, for example, are two major cellulolytic organisms that produce acetate and H_2 as a result of cellulose degradation (Wolin et al. 1997). *Streptococcus bovis* is one of the major starch digesting bacteria in the rumen and produces lactate as a major end product (Kamra 2005). If microbial degradation of substrate in the rumen declines, then feed intake is reduced because forage particles cannot flow out of the rumen. This observation is corroborated by intake data where intake of SS was significantly less than SH and intake of AS was significantly less than intake of AH (Table 4.3). Further to this, silage diets produced less total VFA than hay diets although this effect does not become statistically significant until period three (Table 4.5). A possible explanation for the differences observed in the abundance of bacteria from cattle fed hay compared to silage is that hay is a more available source of structural carbohydrates, thereby enhancing populations of bacterial species that directly and indirectly make use of these compounds.

Fibrolytic microorganisms play a crucial role in methanogenesis and their abundance has been positively correlated with the abundance of methanogens in the rumen of various animals including cattle (Morvan et al. 1996). When fiber is digested and fermented by rumen bacteria, H_2 is produced as a waste end product. Nevertheless, H_2 is the reducing agent that drives the electron transport chain in methanogens and is necessary for their growth and proliferation (Takahashi, 2001). Methanogenic archaea decreased to a greater extent in SS-fed steers compared to SH-fed steers (Figure 4.7). These findings likely resulted from a reduction in the flow of H_2 from prokaryotic bacteria since they were more acutely suppressed in the silage diet. It is not likely that the CT in

the SS diet was directly responsible for depressed methanogen growth as the population continued to decline in period three when steers once again received an AS diet. Accordingly, it is our deduction that any decline observed in the methanogen population was a direct effect of reduced fiber digestion.

Ciliate protozoa account for a large portion of the biomass in the rumen and are highly capable of enhancing methanogenesis (Williams and Coleman 1992). Hydrogen is produced in large quantities within the hydrogenosomes of ciliate protozoa as a fermentation by-product. The resulting H_2 is used by methanogens found living inside or in close association with the protozoal cells and allows the fermentation of organic matter to proceed mainly to acetate and CO_2 at the expense of lactate and butyrate production (Morgavi et al. 2010). Methanogens found inside and attached to ciliate protozoal cells have been estimated to contribute between 9% and 37% of rumen methanogenesis (Finlay et al. 1994; Newbold et al. 1995). Reducing the number of protozoa in the rumen is identified as a possible strategy for decreasing enteric CH_4 formation. In our research, ciliate protozoa populations were enhanced by feeding sainfoin irrespective of preservation technique (Figure 4.8). Similar results were found by Scharenburg et al. (2007), who evaluated the effect of dehydrated and ensiled sainfoin treated with and without PEG on the rumen microbial populations of lambs. In their study, protozoa counts were enhanced by sainfoin-CT when compared against PEG-treated sainfoin feeding. Interestingly, conflicting research conducted by Benchaar et al. (2008) found that total numbers of protozoa were unchanged by the addition of CT from quebracho trees to the diet of lactating cows. Further research is needed to better understand the direct effects of CT on rumen ciliate protozoa. As mentioned earlier in this discussion, Beauchemin et al. (2007) observed that increasing levels of CT linearly decreased total

VFA and increased the proportion of propionate present in the rumen. The enhanced protozoal populations in our research may partially explain why an increase in the molar proportion of propionate was not observed in rumen fluid samples obtained from steers fed CT-containing diets. It is reasonable to hypothesize that in these steers, the fermentation of organic matter proceeded largely to acetate as an end product at the expense of lactate. Lactate is the main intermediate in the conversion of starch to propionate (Moss et al. 2000). From our results, it appears that the relative abundance of methanogens (although not analyzed statistically) is positively correlated with the abundance of protozoa except in the case of sainfoin silage. Prokaryotic bacteria were acutely suppressed in the sainfoin silage diet which may have decreased the amount of H_2 available to methanogens for methanogenesis and subsequently their abundance.

4.5.2 Implications

In spite of its limited use, sainfoin is an acceptable forage source for backgrounding cattle. Throughout our 15-wk trial, steers experienced satisfactory intakes and rates of gain. A significant decline in enteric CH_4 production could only be detected in SH-fed animals compared to AH when expressed as L d^{-1} or L kg BW^{-1}, but not as L kg DMI^{-1} or %GEI. The sainfoin in our diets contained 1% CT on a DM basis which is likely not enough to stimulate reductions in enteric CH_4 production to the extent realized by research conducted in New Zealand (Woodward et al. 2001). To determine if feeding CT-containing plants is a feasible CH_4 mitigation strategy for the Canadian Cattle industry, additional research must be conducted to verify that plants grown in a western Canadian production environment contain sufficient levels of naturally occurring CT.

Method of feed preservation did not appear to have a significant effect on enteric CH_4 emissions from steers. In spite of this, hay diets did lead to more favorable rumen

fermentation profiles for decreased enteric CH_4 formation than silage diets. These observations conflict with Sundstøl's perspective (1981) that ensiling rather than drying forages would lead to reductions in CH_4 production due to decreased digestion in the rumen. More *in vivo* experiments are needed to explore the effects of method of feed preservation on enteric CH_4 production. Additionally, little is known about the consequence of forage preservation on the biological activity of CT. Since CT are predominately localized in the leaves of forages, hay-making may reduce the total CT concentration of forages due to loss of leaf (Scharenberg et al. 2007). Conversely, ensiling may reduce the total CT content of feeds or alter the distribution of extractable versus bound tannin (Scharenberg et al. 2007). Although the SS and SH diets in our research contained similar concentrations of CT, it is possible that the content of bound CT was higher in the SS. It has been suggested that bound CT are inert and do not affect microbial fermentation (Makkar and Becker 1997). If the CT in our SS remained bound, they would not have interacted with bacterial populations in the rumen. This may partially explain why a decrease in enteric CH_4 production could not be detected in SS-fed steers as compared to AS-fed steers. More research is needed to evaluate the effect of method of forage preservation, particularly ensiling, on the persistence of CT and their subsequent effect on enteric CH_4 formation.

Terminal restriction fragment length polymorphisms data indicates that the archael population of the rumen is stable and minutely affected by diet, animal performance parameters and or ambient temperature. Consumption of a CT-containing diet does not appear to have a major affect on the relative abundance of rumen prokaryotic bacteria or methanogens. The apparent lack of effect may be attributed to the relatively low concentration of CT (1%) in the sainfoin diets. Real time-PCR data does demonstrate that

the relative abundance of rumen prokaryotes is lower in silage diets than in hay diets. As well, methanogenic archae were limited to a greater extent in the SS-fed steers compared to SH-fed steers. It is probable that the depressed methanogen growth observed in our experiment is a result of reduced H_2 availability resulting from decreased fiber digestion. Future research should explore the possibility of reducing methanogenesis without altering fiber degradability in the rumen by shifting the balance between H_2 producers (*Ruminococci*) and non-H_2 producers (*Fibrobacter*) in the fibrolytic community. Interestingly, the relative abundance of ciliate protozoa populations was enhanced by feeding sainfoin irrespective of preservation technique. Further research is needed to better understand the direct effects of CT on rumen ciliate protozoa as results on this subject are varied (Scharenberg et al. 2007; Benchaar et al. 2008).

5.0 GENERAL DISCUSSION

Enteric CH_4 production from cattle represents a significant contribution to Canada's greenhouse gas inventory as well as an inefficiency in meat production since large portions of ingested energy are lost as eructated CH_4. Decreased enteric CH_4 formation would reduce the carbon footprint of cattle and improve feed efficiency. Research suggests feeding CT at concentrations ranging between 2-4% of forage DM elicits positive responses in cattle performance (Aerts et al. 1999) including decreased enteric CH_4 production, improved protein digestion, reduced incidence of bloat and internal parasite control. In western Canada, legumes such as sainfoin and birdsfoot trefoil naturally contain CT and are of interest to the beef cattle industry for use as feed.

Condensed tannins are considered inhibitory to the growth of rumen bacteria because of their ability to complex with polymers and minerals. It is hypothesized that CT may affect rumen methanogenesis directly by inhibiting the growth of methanogens or indirectly by reducing fiber digestion and decreasing H_2 production in the rumen (Tavendale et al. 2005). Decreased H_2 in the rumen is detrimental to methanogen growth and proliferation as it is reducing agent that drives the electron transport chain in methanogens (Takahashi 2001).

Method of feed preservation (silage versus hay) may provide an additional avenue for reducing enteric CH_4 formation from cattle. Research suggests that extensive fermentation incurred during the ensiling process decreases digestion in the rumen and consequently reduces CH_4 production as a %GEI (Sundstøl 1981).

In this study, 40 yearling steers were used to analyze the effect of sainfoin grown in a Manitoba production environment and preserved as round bale silage/hay on enteric

CH_4 production, rumen fermentation characteristics and rumen bacterial ecology during a western Canadian winter.

Sainfoin diets were well received by the yearling steers as animals experienced acceptable intakes and did not appear to have issues with the palatability of their feed. Further to this, steers receiving the SS and SH diets gained 0.7 and 0.6 kg d^{-1} respectively (Table 4.3.) and did not differ from those steers receiving alfalfa diets. It is possible that cold temperatures experienced during periods one and two suppressed steer weight gain as a consequence of increased maintenance demands. Improved rates of gain were observed in steers during period three and may be linked to warmer ambient temperatures experienced at that time.

A significant decline in enteric CH_4 production could only be observed in SH-fed animals compared to AH (Table 4.4.) when expressed as L d^{-1} or L kg BW^{-1}, but not as L kg DMI^{-1} or as a %GEI. Our sainfoin diets contained 1% CT on a DM basis. As mentioned earlier, beneficial effects of CT on ruminant animal performance, including reduced enteric CH_4 production, are typically experienced at concentrations between 2-4% DM (Aerts et al. 1999). Additionally, sainfoin contains high MW CT which have been observed to precipitate less protein per unit weight than CT with low MW. For this reason, CT originating from sainfoin may need to be fed at slightly higher concentrations than low MW CT to achieve a beneficial response in ruminant animal performance. It is possible that the sainfoin diets in our research did not contain sufficient quantities of naturally occurring CT to stimulate reductions in enteric CH_4 emissions when expressed as L kg DMI^{-1} or as a %GEI.

Method of feed preservation does not appear to have a significant effect on enteric CH_4 production although it does appear to influence rumen fermentation. Hay-fed diets

largely resulted in higher quantities of total VFA production as well as lower acetic:propionic acid ratios however this effect does not become significant until period three. Increased proportions of propionate in the rumen are associated with reduced enteric CH_4 formation because propionate production is an alternative pathway for the use of H_2 (Mathison et al. 1998). Although reductions in CH_4 were not detected, favorable changes in rumen fermentation did occur.

It is largely accepted that cattle fed forage will lose 6% of their ingested energy as eructated CH_4 (Johnson and Johnson 1995). It should be noted that the cattle in our research, with the exception of one group of steers in period three, experienced CH_4 emissions well below 6% of their GEI (Table 4.4.). It is possible that cold environmental temperatures (Figure 4.2.) experienced during our research trial are responsible for the lower than expected emissions. Increased ruminal passage rate constants of fluid and particulate matter have been reported in cold-adapted animals (Kennedy and Milligan 1978). In turn, increased rate of particle passage through the rumen is correlated with decreased CH_4 production (McAllister et al. 1996). Accordingly, the freezing temperatures routinely experienced during western Canadian winters may naturally mitigate CH_4 production in cattle.

Cluster analysis indicates that no changes occurred in the rumen archaeal community structure of experimental animals. This is surprising since half the steers were fed CT-containing forages for a considerable period of time. Our data suggests that the archaeal population of the rumen is stable and minutely affected by diet or ambient temperature.

Relative abundance data from RT-PCR showed that silage diets inhibited far more rumen microbial species than did hay diets. A possible explanation for the differences

observed in the abundance of prokaryotic bacteria from cattle fed hay compared to silage is that hay is a more available source of structural carbohydrates, thereby enhancing populations of prokaryotic species that directly and indirectly make use of these compounds. Accordingly, method of feed preservation does have an effect on bacterial abundance.

Methanogens were less abundant in the rumen fluid samples of steers fed SS than in those fed SH. It is unlikely that the abundance of methanogens from SS-fed animals decreased as a direct result of CT but rather a reduction in the flow of H_2 since prokaryotic bacteria were less abundant in the silage diets. Hydrogen is produced as a waste end product when fibrous feeds are digested by rumen bacteria and is necessary for methanogen growth and proliferation (Takahashi, 2001).

Irrespective of preservation technique, ciliate protozoa populations were enhanced by feeding sainfoin. These observations are consistent with research performed by Scharenburg et al. (2007) but inconsistent with research performed by Benchaar et al. (2008). Further research is required to better understand the effects of CT on rumen ciliate protozoa.

Results from this study are helpful in understanding the effects of CT on rumen bacterial ecology and it's correlation with enteric CH_4 production. Our observations indicate that rumen methanogens are resilient to changes in diet and temperature. Research efforts should focus on the effect of CT on fiber degrading bacteria since suppression of H_2 formation in the rumen appears to be the most effective means to alter enteric CH_4 formation.

6.0 CONCLUSIONS

It can be concluded that:

- Sainfoin grown in a Manitoba production environment and preserved as round bale silage/hay effectively supported the maintenance and growth of backgrounding steers during a western Canadian winter.
- A significant decline in enteric CH_4 production could only be observed in SH-fed animals compared to AH when expressed as L d^{-1} or L kg BW^{-1} but not as L kg DMI^{-1} or %GEI.
- Method of feed preservation (silage versus hay) does not appear to have a significant effect on enteric CH_4 production from steers fed alfalfa diets.
- Method of feed preservation (silage versus hay) does appear to have an effect on rumen fermentation. Hay diets largely resulted in higher quantities of total VFA production as well as lower acetic:propionic acid ratios.
- A significant decline in enteric CH_4 production was not observed in SS-fed animals compared to AS even when expressed as L d^{-1} or L kg BW^{-1}. This absence of an effect may be a consequence of a possible shift in the distribution of CT resulting from the ensiling process. Further studies should be performed to gain a better understanding on the effect of method of feed preservation on CT-containing legumes.
- The methanogen population of the rumen appears stable and is minimally affected by diet, animal performance parameters (intake (% BW), CP intake, GE intake, CT intake and CH_4 (%GEI)) and ambient temperature in this experiment.

- Methanogens were less abundant in the rumen fluid samples of steers fed SS. It is unlikely that this is a direct result of CT but rather a reduction in the flow of H_2 since prokaryotic bacteria were less abundant in the silage diets.
- Bacterial populations, excluding *Lactobacillus species*, were more plentiful in hay diets. This is likely because hay is a more available source of structural carbohydrates than silage. Further research is required to investigate the effect of method of feed preservation on rumen microbes.
- Sainfoin diets enhanced the abundance of ciliate protozoa. These results are consistent with research performed by Scharenburg et al. (2007). Further research is required to better understand the effects of CT on rumen ciliate protozoa.
- Further research must be conducted to completely understand of the effect of CT on rumen microbial ecology and subsequent effects on enteric CH_4 formation.

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8.0 APPENDIX

Table 8.1 Contrasts to show diet x period interactions for methane production

Item	Significance of effect[z]		
	1 vs 2[y]	1 vs 3	2 vs 3
CH_4 (L day^{-1})			
pen 1 vs pen 2	NS	NS	NS
pen1 vs pen 3	NS	NS	*
pen 1 vs pen 4	NS	NS	**
pen 2 vs pen 3	NS	*	NS
pen 2 vs pen 4	NS	NS	*
pen 3 vs pen 4	NS	NS	NS
CH_4 (L kg BW^{-1})			
pen 1 vs pen 2	NS	NS	NS
pen1 vs pen 3	NS	NS	NS
pen 1 vs pen 4	NS	NS	*
pen 2 vs pen 3	NS	NS	NS
pen 2 vs pen 4	NS	NS	*
pen 3 vs pen 4	NS	NS	NS
CH_4 (L kg DMI^{-1})			
pen 1 vs pen 2	NS	NS	NS
pen1 vs pen 3	NS	NS	*
pen 1 vs pen 4	NS	NS	**
pen 2 vs pen 3	NS	NS	NS
pen 2 vs pen 4	NS	NS	*
pen 3 vs pen 4	NS	NS	NS
CH_4 (% GEI)			
pen 1 vs pen 2	NS	NS	NS
pen1 vs pen 3	NS	NS	*
pen 1 vs pen 4	NS	NS	**
pen 2 vs pen 3	NS	NS	NS
pen 2 vs pen 4	NS	NS	*
pen 3 vs pen 4	NS	NS	NS

[z]Significance: *P < 0.05; **P < 0.01; NS = not significant

[y]1 vs 2 = contrasts of least square means in period 1 with period 2

Table 8.2 Contrasts to show diet x period interactions for rumen fermentation characteristics

	Significance of effect[z]		
Item	1 vs 2[y]	1 vs 3	2 vs 3
Acetic acid			
pen 1 vs pen 2	NS	***	***
pen1 vs pen 3	NS	*	*
pen 1 vs pen 4	NS	*	NS
pen 2 vs pen 3	NS	***	***
pen 2 vs pen 4	NS	***	***
pen 3 vs pen 4	NS	***	***
Propionic acid			
pen 1 vs pen 2	*	***	***
pen1 vs pen 3	NS	NS	*
pen 1 vs pen 4	*	***	**
pen 2 vs pen 3	NS	***	***
pen 2 vs pen 4	NS	**	**
pen 3 vs pen 4	NS	***	***
Acetic:Propionic			
pen 1 vs pen 2	***	***	NS
pen1 vs pen 3	***	*	*
pen 1 vs pen 4	***	*	NS
pen 2 vs pen 3	NS	*	NS
pen 2 vs pen 4	*	*	NS
pen 3 vs pen 4	NS	NS	NS
Butyric acid			
pen 1 vs pen 2	NS	***	***
pen1 vs pen 3	NS	NS	*
pen 1 vs pen 4	NS	***	***
pen 2 vs pen 3	NS	***	***
pen 2 vs pen 4	NS	NS	*
pen 3 vs pen 4	NS	***	***
Total VFA			
pen 1 vs pen 2	NS	***	***
pen1 vs pen 3	NS	NS	**
pen 1 vs pen 4	NS	***	*
pen 2 vs pen 3	NS	***	***
pen 2 vs pen 4	NS	***	***
pen 3 vs pen 4	NS	***	***

[z]Significance: ***P < 0.001; **P < 0.01; *P < 0.05; NS = not significant

[y]1 vs 2 = contrasts of least square means in period 1 with period 2