2014

Reduction Of Enteric Methane Production: A Nutritional Approach

A'ja V. Duncan
North Carolina Agricultural and Technical State University

Reduction of Enteric Methane Production:

A Nutritional Approach

A'ja V. M. Duncan

North Carolina A&T State University

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department: Energy and Environmental Systems

Major: Energy and Environmental Systems

Major Professor: Dr. Abraham Woldeghebriel

Greensboro, North Carolina

2014

The Graduate School
North Carolina Agricultural and Technical State University
This is to certify that the Doctoral Dissertation of

A'ja V. M. Duncan

has met the dissertation requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2014

Approved by:

Dr. Abraham Woldeghebriel Major Professor	Dr. Zerihun Assefa Committee Member
Dr. Keith Schimmel Committee Member	Dr. Mulumebet Worku Committee Member
Dr. Keith Schimmel Department Chair	Dr. Sanjiv Sarin Dean, The Graduate School

Biographical Sketch

A'ja Vanessa Moore Duncan was born in El Paso, Texas to Ronnie Moore and the late Vanessa A. Moore. She is a wife to Eric and the mother of two wonderful children, Elijah and Erin. She was crowned Ms. Iota Phi Lambda in 2000 in Winston-Salem, NC. She earned a Bachelor of Science degree in Chemistry from North Carolina Central University in Durham, NC. With funding from a Louis Stokes Alliance for Minority Participation Fellowship, she was an intern for the North Carolina Department of the Environment and Natural Resources (Radiation Protection Safety), where she implemented a plan for North Carolina to work with all U.S. States and territories to safely dispose of radioactive waste in North Carolina. She was an Environmental Careers Organization Intern at the U.S. EPA in Research Triangle Park, NC. She later became a student services contractor in the Methods Development and Applications Branch in the Office of the National Exposure Research Laboratory at the U.S. EPA, where she worked on method applications to detect pesticides, heavy metals, and the Consolidated Human Activity Database. A'ja received a Master of Science degree in Chemistry concentrating in Analytical Chemistry from North Carolina Central University, where she completed her thesis work in the Department of Environment and Geospatial Sciences using Geographical Information Systems (GIS) to determine the impact of land use on stream water quality in the Upper Cape Fear and Neuse River Basins. She was a part-time Organic Chemistry Instructor at Shaw University. While attending North Carolina A&T State University in Greensboro, NC in the Energy and Environmental Systems Department, she was supported by a USDA National Needs Fellowship. She participated in the NASA Guilford County Schools summer program to assist teachers in developing lesson plans for integrating NASA research in the classroom.

Dedication

I dedicate this work to my husband Eric, my children Elijah, Erin, and my mom (grandma) Shirley for their continued support to accomplish this challenging yet rewarding task.

Acknowledgements

This PhD has been a journey of laughs, tears, and dedication. I appreciate the journey, and I look forward to the opportunities that this experience will lead to in my future. I would like to thank God for giving me the strength to take on this task with the many obstacles that I have faced. I would like to give thanks and appreciation to my advisor Dr. Abraham Woldeghebriel. I would like to give thanks and appreciation to my committee members Dr. Zerihun Assefa and Dr. Mulumebet Worku. I would like to acknowledge the USDA National Needs Fellowship (Award No: 2010-38420-20422) for funding me.

I would like to thank the Department of Energy and Environmental Systems Faculty and Personnel for support to complete my research. I would also like to express my gratitude to the School of Agriculture and Environmental Sciences and the Department of Animal Sciences Faculty and Staff for their support in completing my research. A special thanks to Dr. Woldeghebriel for his continued guidance, patience, and knowledge for introducing me to a new field in research that has allowed me to apply my background in Chemistry to understand ruminant nutrition. I would like to express my gratitude to Dr. Schimmel for his continued support with funding and supplies for my research throughout this journey. A special thank you to Dr. Assefa for his willingness to always assist in my research and providing supplies to make it a success. I would to express my gratitude to Dr. Worku for being a great mentor who always provided encouragement and guidance. I would like to thank Mr. Hamid Ismael for his statistical analysis for real-time PCR and his assistance with PCR techniques.

This research was made possible by the North Carolina Department of Agriculture and Consumer Sciences. I would like to give thanks to NC Department of Agriculture Administration and specifically Joe Hampton for the approval of the use of the animals in my research. Also a

special thanks to the Piedmont Dairy Research Station Management team for the use and care of the animals provided for the study and for always willing to assist me to make this research possible. I would like to thank the NC A&T School of Agriculture Analytical Services Laboratory, specifically Mr. John Carver and Mr. Bryce Holmes, for sample analytical analysis and allowing me to watch and learn. I would like to thank the Department of Chemistry, specifically Mr. James King, for assisting with the mineral analysis. I would like to thank the Department of Family and Consumer Sciences and specifically Ivy Smith for protein analysis. Appreciation to Dr. James Henson, Southern University, for help with the statistical analysis. I appreciate Dr. Saundra DeLauder for her continued guidance and pushing me to reach my goals. I would like to thank Mr. Ahmed Alshareef for the help with techniques in DNA isolation. I would like to give thanks to Jamie Williams, a friend, for her continued support in and out of the laboratory, especially with understanding real-time PCR. I would like to give thanks to Andrea Byers for her continued support and guidance in PCR techniques. I would like to thank all my family and friends that supported me through this memorable journey. A special thank you is given to my husband Eric Duncan for encouraging me to complete this dissertation. I would like to thank all of my family and friends for their support. Also, I would like to give a special thanks to Elijah and Erin Duncan for the smiles that have given me the drive to complete this program.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADF- acid detergent fiber

ADP- adenosine di-phosphate

ATP- adenosine tri-phosphate

BLAST- Basic Local Alignment Search Tool

bp - base pair

BW- body weight

CH_4- methane

CO_2- carbon dioxide

DM- dry matter

DMI-dry matter intake

EPA- Environmental Protection Agency

Exp. – experiment

F-forward primer

FIA-Flow Injection Analyzer

g- gram

GC- gas chromatography

GEI – gross energy intake

GHG(s) - greenhouse gas (es)

GWP- global warming potential

H_2- hydrogen gas

H_2O- water

ICP-MS- Inductively Coupled Plasma-Mass Spectrometry

IPCC-Intergovernmental Panel on Climate Change

Kg- kilogram(s)

L-liter

LY- last year feed

m- Meter

mg - milligram

mL -milliliter

mm – millimeter

NCBI-National Center for Biotechnology Information

NCDA&CS- North Carolina Department of Agriculture and Consumer Services

aNDF- neutral detergent fiber

NH_3- ammonia

NO_3- nitrate

OM- organic matter

PCR- polymerase chain reaction

ppm - parts per million

PS- particle size

p-value- probability value

R-reverse primer

TMR- total mixed ration

TY- this year's feed

UNFCCC- United Nations Framework Convention on Climate Change

VFA(s) - volatile fatty acid(s)

Abstract

This study investigated the effects of feed particle size and nitrate or fumarate alone or in combination on *in vitro* fermentation, abundances of methanogens, and methane production. Rumen fluid was collected from a Holstein-Friesian dry cow and a steer (experiment 1) and a dry cow and two steers (experiment 2) averaging 625.4 kg in body weight (BW). The cattle were offered 11.4 kg/d per animal of concentrate diet containing equal amounts of soybean meal, whole cottonseed, and ground corn once a day with grass hay at *ad libitum* (experiment 1) and 15.9 kg/d per animal of total mixed ration (TMR) of silage, hay, corn, corn gluten, soybean meal, and minerals (experiment 2). Feed grab samples were collected, oven-dried, coarsely pulverized in a regular kitchen blender for one minute, and separated into three particle sizes (PS: 0.85, 1.4, and 2.36 mm). The feed additives used were nitrate, fumarate, and a nitrate-fumarate mixture. The fermentation parameters measured after 48h incubation periods were methane (CH_4), pH, VFA, nitrate (NO_3), and ammonia (NH_3). Real-time PCR was used to quantify the relative abundances of total and specific methanogens.

The results of the study revealed that the addition of fumarate had no effect on CH_4 production. The addition of nitrate reduced CH_4 production ($p < 0.05$) by 57% and 59% in experiments 1 and 2, respectively. The addition of the nitrate-fumarate combination also reduced ($p < 0.05$) CH_4 production by 40 % (experiment 1) and 68% (experiment 2). Methane production was affected by feed particle size. In experiment 1, CH_4 production for the medium PS was 31% and 39% lower ($p < 0.05$) than the small and large PS, respectively. However, in experiment 2 CH_4 production was 17% and 16% higher ($p < 0.05$) for the large PS compared to the small and medium PS, respectively. The addition of feed additives had no effect on total methanogens, while fumarate decreased ($p < 0.05$) the abundance of *Methanobrevibacter* sp. AbM4 and nitrate

decreased ($p < 0.05$) the abundance of *Methanosphaera stadtmanae*. Nitrate reduced ($p < 0.05$) individual and total VFAs in experiments 1 and 2, while fumarate increased ($p < 0.05$) propionate and decreased ($p < 0.05$) butyrate, valerate, and iso-valerate compared to the control (experiment 2). Fumarate had no effect on total VFA production in experiments 1 and 2. In experiment 1, the nitrate-fumarate combination reduced ($p< 0.05$) butyrate and iso-butyrate compared to the control and had no effect on other individual VFAs. The addition of the nitrate-fumarate combination decreased ($p< 0.05$) the production of acetate, butyrate and total VFAs (experiment 2). In experiment 1, addition of fumarate increased ($p < 0.05$) pH, but there was no effect in experiment 2. The addition of nitrate and nitrate-fumarate combination increased ($p < 0.05$) pH in both experiments. The addition of nitrate also increased ($p < 0.05$) the concentration of nitrate and ammonia. On the other hand, addition of fumarate and the nitrate-fumarate combination had no effect on nitrate and ammonia concentrations.

In summary, nitrate alone or in combination with fumarate was effective in reducing *in vitro* methane production. The use of nitrate also reduced VFA production but when combined with fumarate, the reduction in VFA production was lessened, indicating that addition of fumarate to nitrate not only reduces methane production but also seem to spur the reduction of VFAs by nitrate. The effects of the individual feed additives appear to be very specific but when used in combination they seem to be more effective in reducing *in vitro* methane production. In conclusion, the mechanism by which nitrate alone or in combination with fumarate reduces methane production is less clear and the most likely scenario could be due to the direct effect on the methanogens or through the reduction of organic matter fermentation that lowers availability of free H_2 for methane production.

Keywords: *methane, VFA, nitrate, fumarate, in vitro, methanogens*

CHAPTER 1

Introduction and Literature Review

1.1 General Introduction

Greenhouse gases (GHG) have the ability to trap heat in the atmosphere. Greenhouse gases occur in the atmosphere naturally and from anthropogenic sources. The primary GHGs are carbon dioxide (CO_2), methane (CH_4), ozone (O_3), and nitrous oxide (N_2O) with additional anthropogenic GHGs such as halocarbons, hydrofluorocarbons, and perfluorocarbons (IPCC, 2007). The GHG effect is caused by GHGs absorbing infrared radiation from the Earth's surfaces and in turn that radiant heat is emitted from the earth's atmosphere and is trapped within the surface-troposphere (IPCC, 2007) with the GHG acting as a blanket. Climate change can occur due to changes in the atmosphere and one of these changes can be due to increased GHG concentrations.

Even though GHGs occur naturally, there has been a significant increase in their concentrations over the last 250 years due to human activities (IPCC, 2007). Therefore, the United Nations Framework Convention on Climate Change (UNFCCC) was adopted in 1992 to control global climate change by encouraging countries to stabilize GHG emissions (UNFCCC, 2012). The UNFCCC acknowledges that human activities increase the concentration of GHGs in the atmosphere, which results in global warmer surface temperatures. Therefore, the UNFCCC adopted the Kyoto protocol in 1997 which committed industrialized countries, like the United States, to reduce GHGs emissions (UNFCCC, 2012).

1.1.1 Sources of Greenhouse Gases. The primary sources of GHGs in the United States are electricity production, transportation, industry (burning fossil fuels), agriculture, land use and

forestry. Greenhouse gas emissions from agriculture have increased by approximately 13% since the 1990s (EPA, 2012). In 2010 agriculture accounted for nearly 7% of the U.S. GHGs emissions (EPA, 2012) and globally agriculture accounted for 14% of GHG emissions (IPCC, 2007). The IPCC categorizes GHGs into two categories: short and long-lived GHGs. An example of short-lived gases is carbon monoxide (CO) because it can be removed by natural oxidation in the environment. Long-lived GHGs like CH_4 remains in the atmosphere for over a decade and can have long-term effects on climate change.

1.1.2 Methane. Methane is a greenhouse gas that is colorless and odorless. Methane contributes to global warming and climate change. Methane is produced in the rumen by a group of Archaea known as methanogens (Hook, Wright, & McBride, 2010). Methanogens produce CH_4 as a part of the normal process of feed digestion in ruminants. Domesticated animals have been identified as major producers of CH_4. Methane that is exhaled from the ruminant animal is a loss of feed-derived energy to the animal and this loss of CH_4 to the atmosphere varies based on ruminant species (Hook et al., 2010).

Methane has a relative effectiveness 21 times higher than CO_2 and a 12 year atmospheric lifetime (IPCC, 2007). For this reason CH_4 is a significant contributor to global warming and climate change (Johnson & Johnson, 1995). Climate change can have many undesirable consequences including rises in sea level, warmer temperatures, changes in water quality, increased exposure to diseases that affect humans and animals, alteration of the number of pests that affect plants, but most importantly significant economic costs in agriculture (Moss, Jouany, & Newbold, 2000). Anaerobic environments (natural or man- made such as waste processing) are among the major source of biological CH_4 emitters. Agriculture is recognized as a significant contributor to GHGs with CH_4 being the most popular on that list.

One of the agricultural activities that contribute to atmospheric CH_4 concentrations is enteric fermentation from ruminant animals. Diet plays a major role in the variability of CH_4 loss to the animal and there are two major mechanisms, the rate and passage of fermentable carbohydrate and hydrogen supply in relation to volatile fatty acids (VFA) production. The ratio of propionic acid produced relative to acetic acid impacts the production of CH_4 in the rumen (Johnson & Johnson, 1995). Cattle eructate CH_4 during the digestion of feed, and it is estimated to be approximately 6% loss of the gross energy intake of the animal (Johnson & Johnson, 1995). The eructation of CH_4 begins in cattle shortly after solid feeds are introduced into their diet. The production of CH_4 from ruminants is inevitable and since CH_4 emissions has a direct and indirect impact on climate change (Johnson & Johnson, 1995), then strategies to reduce the contribution of CH_4 from ruminants is of great interests.

1.1.3 Ruminant Animals. The stomach of a ruminant animal consists of four compartments: the rumen, reticulum, omasum, and abomasums. Ruminant animals can digest coarse plant material. Ruminant and pseudo-ruminant animals have large anaerobic fermentative chambers located at the beginning of their digestive tract (reticulo-rumen) and are much more efficient for the digestion and degradation of plant cell walls. The rumen is home to a number of microorganisms like bacteria, protozoa, and fungi that break down plant materials into simple sugars that the animal can utilize for energy. The formation of glucose from fermentation of feedstuffs is an anaerobic oxidative process that occurs in the Embden-Meyerhof- Parnas pathway. This process forms reduced co-factors like nicotinamide adenine dinucleotide hydride (NADH). These reduced cofactors are re-oxidized to NAD^+ to complete the fermentation of sugars and is regenerated by electron transfer to proton acceptors other than oxygen like CO_2, sulphate, nitrate, and fumarate (Moss et al., 2000). Microbial electron transport-linked

phosphorylation is a way of generating ATP from the flow of generated electrons through membranes. However the process that produces CH_4 represents an inefficient loss of this feed energy (Moss et al., 2000).

According to EPA (2012) ruminant livestock account for 20% of the U.S. CH_4 emissions. In 2009 enteric fermentation was ranked second to natural gas systems for the production of CH_4 (EPA, 2012). It has been noted by EPA (2012) that cattle are the major producers of CH_4 with beef cattle ranked above dairy cattle. Enteric CH_4 emission from ruminants is of great interest in agricultural and environmental research. There are several studies (Beauchemin & McGinn, 2006b; Boadi, Benchaar, Chiquette, & Masse, 2004; Iqbal, Cheng, Zhu, & Zeshan, 2008) that suggest the acetate production contributes to a higher proportion of CH_4 production and while higher proportions of propionate results in a significant reduction in CH_4 production (Beauchemin & McGinn, 2006b; Boadi et al., 2004; Iqbal, Cheng, Zhu, & Zeshan, 2008).

1.2 Literature Review

1.2.1 Nutritional and Mitigation Strategies used to Reduce Enteric Methane.

Methane production in the rumen can be altered by different strategies like the use of various feed additives that can act as rumen modifiers. Current research implies that factors like feed intake, forage type, forage processing, and rumen microbial manipulation all have an impact on the amount of CH_4 produced by ruminants. There are many mitigation strategies that focus on reducing or inhibiting methane emissions by manipulating the rumen environment. Improving the efficiency of animals by investigating biological traits that improve production systems can reduce the impact that animals have on the environment (Bell, Wall, Russell, Simm, & Stott, 2011). One of the major substrates that methanogens utilize to reduce CO_2 to CH_4 is hydrogen (Hook et al., 2010). Therefore, introducing feed additives into the ruminant diet that can

generate H_2 utilizing reactions that alter the rumen to reduce the concentrations of H_2 is one of the means researchers have proposed that may reduce the ability of methanogens to produce CH_4.

1.2.1.1 Fats and Essential Oils. Fats are commonly added to dairy cattle diets for energy, maintenance and milk production (Boadi et al., 2004). The addition of fats to the diet has been shown to reduce CH_4 emissions. However there are variations in the reduction of CH_4 and this may be due to the type of fat used as a feed additive (Boadi et al., 2004). Long-term or excessive use of fats in dairy cattle may result in lower acetate production, which impacts milk fat content.

The addition of fats and essential oils to the ruminant diet impacts CH_4 production by several mechanisms that involve a decrease in acetate concentration, inhibition of protozoa and methanogens in the rumen, and bio-hydrogenation of fatty acids to reduce the availability of free H_2 for CH_4 synthesis (Boadi et al., 2004). Commercially available feed additives like sunflower and canola oils have also been shown to reduce CH_4 production per unit of gross energy intake in beef cattle fed high forage diets due to a depression in digestibility of feed (Beauchemin & McGinn, 2006a). However, sunflower oil was more effective than canola oil in reducing CH_4 production indicating that fatty acid composition may play a role in the mechanisms stated above for which essential oils can decrease CH_4 production (Beauchemin & McGinn, 2006b).

Spanghero, Zanfi, Fabbro, Scicutella, and Camellini, (2008) indicated that in dairy cows the addition of essential oils like oregano, cinnamon, thyme, and orange peel decreased rumen pH which also decreased acetate production and the acetate to propionate ratio. The increase in propionate production is a benefit by the addition of essential oils (Spanghero et al., 2008), but dairy cows need more acetate than propionate for milk fat synthesis. Oilseeds like whole

cottonseed are high in energy and protein and they have been shown to reduce CH_4 and ammonia production, while decreasing protozoal and methanogen numbers over a 12 week period in dairy cows on forage and cereal diets (Grainger, Williams, Clarke, Wright, & Eckard, 2010). Milk yield was also decreased by the addition of whole cottonseed (Grainger et al., 2010). The efficacy of some sources of fat like medium-chain fatty acids to reduce methanogenesis can be related to the diet of the animal (Machmüller, 2006). The disadvantages to the use of fats in the diet include a decrease in fiber digestibility, lower acetate production and decreases in milk yield (Beauchemin & McGinn, 2006b; Iqbal et al., 2008; Spanghero et al., 2008; Grainger, Williams, Clarke, et al., 2010; Grainger & Beauchemin, 2011), which has a negative impact on animal productivity by reducing energy availability.

1.2.1.2 Defaunation. The elimination of protozoa from the rumen is called defaunation and it has been shown to reduce CH_4 emissions, but this technique is also unfavorable because it decreases fiber digestion and most defaunating agents are toxic to the animal. Ionophores (IOPs) like Monensin have been studied for their ability to improve the efficiency of feed and have also been shown to reduce CH_4 emissions (Moss et al., 2000; Boadi et al., 2004). They can increase propionate production, while reducing gram-positive bacteria and dry matter intake. The uses of IOPs are not widely accepted by consumers because of the concern for developing antibiotic resistant bacteria. It has also been stated that IOPs are short term and microbial adaptation has been reported (Boadi et al., 2004).

There are various mechanisms by which ionophores affect the production of enteric CH_4 this includes decreasing acetate production (Boadi et al., 2004), ciliate protozoa and bacteria depression. Monensin and lasalocid , are widely used as feed additives in dairy cows (Boadi et al., 2004). However, monensin has been shown to have no effect on enteric CH_4 emission and

improvement in milk production (Grainger, Williams, Eckard, & Hannah, 2010) while they can increase propionate and reduce gram-positive bacteria (Grainger, Williams, Eckard, & Hannah, 2010). Even though (Grainger, Williams, Eckard, et al., 2010) found no effect of monensin on CH_4 production (Mwenya et al., 2006) reported that monensin reduced CH_4 production. Even though it has been reported that IOPs reduce CH_4 emissions, there are variations in their ability to reduce enteric CH_4 production (Grainger, Williams, Eckard, et al., 2010). The ability of IOPs to reduce CH_4 emissions were short-lived or had no effect at all.

1.2.1.3 Tannins, Plant Extracts, and Plant Secondary Compounds. The ban on the use of antibiotic growth promoters has led to the exploration of plants and plants extracts with anti-methanogenic properties. Tannins are phenolic compounds found in plants and they have been shown to decrease CH_4 production (Jayanegara, Togtokhbayar, Makkar, & Becker, 2009). Tannins and plant extracts have been shown to reduce or inhibit the number of protozoa present in the rumen and can be responsible for altering the rumen environment and impacting microbial fermentation and methanogenesis (Patra & Saxena, 2010)

Plant secondary compounds like essential oils have been used as possible feed additives to reduce CH_4 emissions and there has been evidence that these compounds have antimicrobial properties (Patra & Saxena, 2010). However, previous studies (Beauchemin & McGinn, 2006b; Patra & Saxena, 2010) have indicated that these natural compounds not only lower the digestibility of nutrients but their effects can be short-lived and can lead to microbial adaptation.

1.2.1.4 Immunization and Genetic Variation. Immunization has also been used as a mitigation strategy to reduce CH_4 emissions and improve animal productivity (Boadi et al., 2004). Most immunization strategies are based on cultured methanogen species however there are many methanogen species that have not been cultured or identified (Whitford, Teather, &

Forster, 2001). Therefore the use of immunizations to reduce CH_4 emissions may work only on certain species.

Due to the fact there are still uncultured methanogens that have not been identified and methanogens possess different enzymes to utilize substrates for their survival in the rumen makes it difficult to come up with a universal immunization. So focusing on genetic variation in breeds can give some insight into the variation in CH_4 production when compared between breeds and within herds (Yan et al., 2010). Yan et al. (2010) investigated 20 studies that included Holstein-Friesian, Norwegian Red, and Jersey-Holstein crosses for the effect of genetic variation on CH_4 production. Energy loss (methane energy) as a proportion of energy intake can be reduced by increasing milk-yield, energetic efficiency of milk production, or by reducing energy expenditure for maintenance (Yan et al., 2010). It was suggested by Yan et al. (2010) that selecting dairy cows with high energy utilization and increasing feeding levels that increase the outflow rate of digesta in the rumen can decrease microbial fermentation time and decrease CH_4 production.

1.2.1.5 Organic Acids. The ever increasing awareness of microbial adaptation to certain strategies has led to research in the use of organic acids as alternative feed additives. The addition of organic acids like fumarate and malate has shown to shift volatile fatty acid profiles in favor of propionate, which limits the availability of H_2 for methanogens to use as a substrate (Boadi et al., 2004). Fumaric acid, an intermediate product in the propionic acid pathway, is reduced to sunccinate and this reaction requires H_2 (Boadi et al., 2004). This pathway gives rise to fumarate as being classified as a potential electron sink. However, Beauchemin and McGinn (2006a) reported that fumaric acid had no effect on CH_4 production in Angus heifers. Malate is also reduced to fumarate and then converted to propionate. The effect of malic acid has been

evaluated in mid-lactation grazing Holstein-Friesian cows on their feed intake and CH_4 emissions (Foley et al., 2009). Foley et al. (2009) reported there was no effect of malic acid on dry matter intake (DMI), chemical composition of feed and *in vivo* CH_4 production. The study by (Foley et al., 2009) concluded that malic acid had no effect ruminal CH_4 or milk production. The reductions of dicarboxylic acids like apartate, malate, and fumarate to propionate is considered as a competitive pathway to methanogenesis (Ellis et al., 2008). The microorganisms that reduce organic acids use H_2 and formate to reduce propionate to sunccinate (Ellis et al., 2008). Therefore the use of organic acids should hold promise to reducing enteric CH_4 formation.

1.2.1.6 Nitrate, Nitrocompounds, and Alternative Hydrogen Sinks. The use of alternative H_2 sinks has been investigated (Anderson & Rasmussen, 1998; van Zijderveld et al., 2010; Hulshof et al., 2012) as rumen modifiers. Hydrogen consuming compounds in the rumen include NO_3, sulphate (SO_4), and oxygen (O_2). When electron acceptors are present methanogens can be outcompeted by denitrifying bacteria and sulfate-reducing bacteria (Anderson & Rasmussen, 1998; van Zijderveld et al., 2010). Although denitrifying and sulfate-reducing bacteria are not dominant microorganisms in the rumen, they can increase if these electron acceptors are present (Morgavi et al., 2010). The utilization of NO_3 is acceptable as a feed additive since microorganisms convert NO_3 to NH_3, which is thermodynamically favorable (Morgavi et al., 2010).

Nitrogen containing compounds (nitrocompounds) have been investigated for their anti-methanogenic effect (Anderson et al., 2010; Božic et al., 2009). These compounds have been proven to be a possible H_2 sinks and can inhibit microbial growth in the rumen. The effects of nitrate and nitrite on methanogenic bacteria have been evaluated (Klüber & Conrad, 1998). Nitrogen oxides can have toxic effects on certain bacteria in mammalian cells and CH_4 inhibition

has been shown in methanogens introduced to nitrate and this includes methanogens in rice fields as well (Klüber & Conrad, 1998)

Nitrate is a compound that has been investigated as a feed additive to reduce or inhibit methanogenesis and several studies on nitrate (Božic et al., 2009; van Zijderveld, Fonken, et al., 2011; van Zijderveld, Gerrits, et al., 2011; Zhou, Yu, & Meng, 2011) have documented that nitrate reduces CH_4 production and improves the conversion of dietary energy into metabolizable energy. Nitrate as a feed additive has no impact on milk energy output (van Zijderveld, Gerrits, et al., 2011) and has been associated with variations in VFA profiles.

Nitroethane was also examined *in vivo* in Holstein steers and there was a decrease in CH_4 production and DMI (Gutierrez-Banuelos et al., 2007). The use of nitrate or nitrocompounds has been shown to reduce substrates for methanogenesis. Nitroethane has also been shown to reduce CH_4 and acetate production, while there was no effect on the acetate to propionate ratio, fumarate and butyrate production in Holstein steers (Gutierrez-Bañuelos et al., 2007). Table 1 summarizes the current mitigation strategies and their impacts on enteric fermentation and CH_4 production.

Table 1

Summary of Selected Mitigation Strategies

Mitigation Strategy	Mode of Action	Impact/Comments
Defaunation	Decrease in protozoa	Temporary reduction and affects digestion
Tannins	Decrease in protozoa	Adaptation by protozoa
Saturated Fatty Acids	VFA shift (increase propionate)	Decrease in DMI
Organic Acids	Hydrogen sink/lower pH	Small effect/must be fed daily/expensive
Nitrate and Sulfate	Hydrogen sin	Toxic intermediates/persistent
Ionophores	VFA shift (propionate)/ reduce gram positive bacteria	Consumer Resistance/ microbial adaptation
Enzymes, yeasts, and probiotics	VFA shift/ Hydrogen sink	Variable results, microbial adaptation
Plant Extracts	Microbial inhibition	Microbial adaptation
Immunization	Microbial inhibition	Additional research required/ limited impact
Short-chain nitrocompounds	Microbial inhibition/ H_2 sink	Additional research required

Source: Boadi et al. (2004); Iqbal et al. (2008)

1.2.2 Techniques for Determining Enteric Methane Production.

1.2.2.1 In Vitro Techniques. The ruminant animal has been studied *in vitro* and *in vivo* to understand their metabolism and nutrient requirements for optimal animal performance (Johnson, 1966). Also ruminants rely heavily on microorganisms for nutritional energy than non-ruminants and this knowledge of their dependence on microorganisms has led to the investigation of these microorganisms that inhabit the rumen. *In vitro* techniques require microorganisms from the host animal of interest unlike *in vivo* techniques. In order for any *in*

vitro technique to be useful it has to be determined by the researcher if the study is to duplicate the biochemical processes in the rumen or simply to understand the activity of microbial populations in response to change (Johnson, 1966). In a review published by Johnson, (1966) it was noted that the main advantage to *in vitro* techniques is the ability to study microbial populations without interference from the host environment. There are two types of systems that can be used for in vitro studies: continuous flow systems to simulate the actual digestive process of the animal or closed systems which are used only to quantify specific processes that involve microorganisms. The disadvantage of continuous flow systems is that they are complex and do not allow multiple experiments. The simple designs of closed systems allow many studies to be conducted in an experiment (Johnson, 1966). The use of *in vitro* techniques to understand the fermentation processes that take place in the ruminant has been widely accepted as a useful quantitative tool.

1.2.2.2 In Vivo Techniques. There are various methods to determine CH_4 emissions from ruminants and they are tracer and enclosure techniques. The quantification of CH_4 is made possible by infrared spectroscopy, gas chromatography, mass spectroscopy, gas and mass chromatography (Johnson & Johnson, 1995). The selection of the technique is dependent on the researcher's objective or overall goals. Individual animal techniques include enclosure techniques like respiration calorimetry involving whole animal chambers, head boxes, and ventilated hoods (Johnson & Johnson, 1995). There are two types of tracer techniques: isotopic and non-isotopic methods. Isotopic methods use hydrogen [^{3}H-] and carbon [^{14}C-] labeled CH_4 that is infused into the rumen and the specific activity of the gas is used to calculate total CH_4 production (Johnson & Johnson, 1995). However one of the limitations of using this method is the difficulty in the preparation of isotopic tracers. A non-isotopic method is the sulfur

hexafluoride (SF_6) gas technique that involves placing the SF_6 gas in the rumen and the air around the mouth and nose of the animal is collected in a evacuated sampling canister and measured by gas chromatography (Johnson & Johnson, 1995). This technique is suitable for grazing animals, but the limitation is that hindgut CH_4 production is not measured by this technique (Boadi & Wittenberg, 2001). In contrast to the tracer techniques other direct measurements of CH_4 production from animals require partial or total enclosure. This technique requires animals to be housed in sealed chambers and CH_4 production is determined from the total air flow through the system and the difference in CH_4 concentrations of air entering and leaving the chamber (Johnson & Johnson, 1995). The limitation to this technique is that it is not suitable for grazing animals.

1.2.2.3 Methane Prediction. *In vitro* studies allows the researcher to estimate CH_4 production in the absence of the animal, but CH_4 production can also be predicted or estimated using equations (Wolin et al., 1960; Johnson & Johnson, 1995; Woldeghebriel et al., 2013). A model developed by Wolin (1960) derived a method to predict CO_2 and CH_4 from the molar distribution of major VFAs. The assumptions to the Wolin model emphasizes that hexoses are considered the major fermentation substrates and the only fermentation products considered are acetate, butyrate, propionate, CO_2 and CH_4. The assumption that only acetate, butyrate, and propionate are produced is based on the use of the most abundant VFAs produced in cows. However the Wolin model does not consider microbial H_2 or non-carbohydrate sources as fermentation substrates. This method has been criticized if it is comparable to *in vivo* fermentation, but it is a useful technique to correlate changes in VFA concentrations to CH_4 concentrations. Also models used to predict CH_4 production have been correlated with rumen digestion parameters like consumed gross energy and methanogenesis, while accounting for

variations in the diets (Holter & Young, 1992). The prediction models for estimating of CH_4 can vary by the type of feed and fermentation sites (Ellis et al., 2008). The discrepancy for these equations can arise due to animal to animal variation, but the stoichiometry of the equations can be derived from *in vitro* studies if VFA production is considered since VFA production can't and are rarely studied *in vivo* (Johnson & Johnson, 1995). Methane prediction models and equations must consider all possible variations and conditions (Ellis et al., 2008).

1.2.3 Methanogens. Methanogens belong to the domain Archaea and the kingdom Eurychaeota. Methanogens are genetically similar to eukaryotes and eubacteria and possess a circular chromosome. Methanogens are divided into the following orders *Methanobacteriales, Methanosarcinales, Methanomicrobiales, Methanococcales,* and *Methanopyrales*. Methanogens are established in the rumen shortly after birth and their densities depend on the diet and their relationships with protozoa quite early in the rumen (Skillman et al., 2004). Methanogens possess unique cofactors that make them a distinct group of microorganisms. These bacteria lack the peptidoglycan in their cell walls and it is replaced by pseudomurien (like gram-positive bacteria), protein, glycoprotein or heteropolysaccharides (Hook et al., 2010;Leahy et al., 2010). The methanogen genome is similar to eubacteria genome with genes arranged in operons (Leahy et al, 2010). Methanogen's RNA is synthesized by a single DNA-dependent RNA polymerase however the structure of their RNA polymerase is complex like eukaryotes (McAllister et al., 1996). *Methanobrevibacter* and *Methanomicrobium* are the two species that are known to be dominant in the reticulo-rumen (Attwood et al., 2011).

Methanogens have unique cofactors: coenzyme F_{420}, which is necessary for the enzymes hydrogenase and formate dehydrogenase (Hook et al., 2010) and the coenzyme M or 2-mercaptoethanesulfonic acid , which is the enzyme that is methylated to produce CH_4. The

methanogens vary in certain characteristics. For instance *Methanobrevibacter ruminantium* is rod shaped and has variable motility, but then *Methanobacterium formicicum*, which is in the same order as Methanobrevibacter, is rod or filament shaped without motility (Hook et al., 2010). The methanogens also vary in which substrates (H_2, CO_2, or formate) (table 2) they use for CH_4 production. Table 2 below lists the characteristics and substrates used by the different methanogens species.

Table 2

Classification of Methanogens

Species	Shape characteristic	Substrates for methane production
Methanobrevibacter ruminantium	rod shaped	hydrogen, carbon dioxide, formate
Methanobacterium formicicum	rod or filament shaped	hydrogen, carbon dioxide, formate
Methanosarcina barkeri	coccoid shaped	hydrogen, carbon dioxide, acetate, methylamines, methanol
Methanosarcina mazeii	coccoid shaped	acetate, methylamines, methanol
Methanomicrobium mobile	rod shaped	hydrogen, carbon dioxide, formate
Methanosphaera stadtmanae		hydrogen, methanol (Fricke et al., 2006)
Methanobrevibacter spp.		hydrogen, carbon dioxide

Source: Hook et al. (2010) and Fricke et al. (2006)

The process by which enteric CH_4 is produced in the rumen is known as methanogenesis which is a result of microbial fermentation. The primary microbial fermentation process involves several microbial species including ciliate protozoa, cellulolytic bacteria, and fungi

(Hook et al., 2010). Methanogens are known to be associated with the terminal step in the rumen fermentation process (Hook et al., 2010). Once these plant cell walls and proteins are broken into simple sugars they are further fermented into VFAs, Hydrogen, and CO_2. The methanogens in the rumen mainly produce CH_4 from CO_2 and H_2. The production of acetate produces formate, which is also used as a substrate for methanogenesis (Hook et al., 2010). A study by Whitford et al. (2001) indicated that the largest groups of methanogens in the bovine rumen were *Methanobrevibacter ruminantium* in lactating dairy cattle fed total mixed ration, followed by *Methanosphaera stadtmanae*. *Methanosarcina barkeri* and *Methanobrevibacter* spp. have also been identified in grazing cattle kept indoors and fed total mixed ration (Whitford et al., 2001).

Methanobrevibacter has been detected to be the dominant group of methanogens in the bovine rumen followed by *Methanosphaera stadtmanae* (Skillman et al., 2006). *Methanomicrobium mobile* and *Methanobacterium formicicum* have also been isolated from grazing cattle (Hook et al., 2010). The importance of understanding methanogens is linked to reducing enteric CH_4 emissions. This has led to the whole genome sequencing of *Methanobrevibacter ruminantium* M1 (Leahy et al., 2010). Many enzymes involved in the methanogenesis pathway are conserved and found only among methanogens. The M1 genome is useful in providing details about the methanogenesis pathway in methanogens (Leahy et al., 2010). The M1 genome sequence has revealed that this prominent methanogen has a hydrogenotrophic lifestyle (Leahy et al., 2010), which supports the idea that H_2 is the preferred substrate specific for their metabolism, however the use of other substrates have been identified. Methanogens from various anaerobic habitats have been isolated and studied, but the main focus has been on a selected few dominant methanogens in the rumen (McAllister et al. 1996). A large

part of archaeal populations in the rumen is made up of methanogens (Janssen & Kirs, 2008). Methanogens use the reductive intermediates formyl, methenyl, methylenyl, and methyl to convert CO_2 to CH_4 in a series of steps (McAllister et al., 1996). This process is illustrated below (Figure 1) how methanogens use H_2 as an energy source and the main product from this process is CH_4.

CO_2

↓

Formyl-MF

↓

Formyl-H_4MPT

↓

Methenyl-H_4MPT

↓

Methylene-H_4MPT

↓

Methyl-H_4MPT

↓

Methyl-S-CoM

↓

CH_4

Figure 1. Process of Methanogenesis in Methanobrevibacter ruminantium M1. (Attwood et al., 2011; Leahy et al., 2010)

Methanogens are found in rumen fluid, on feed particles, attached to protozoa, and the rumen epithelium (Janssen & Kirs, 2008). Finding them in various locations may indicate that they could have different growth and passage rates from the rumen. Cultured methanogens have been assigned to species: *Methanobacterium formicicum, Methanobacterium bryantii, Methanobrevibacter ruminantium, Methanobrevibacter millerae, Methanobrevibacter olleyae, Methanomicrobium mobile, Methanoculleus olentangyi*, and *Methanosarcina spp* (Janssen & Kirs 2008). Also *Methanobrevibacter smithii* has been reported as being isolated from the rumen (Janssen & Kirs 2008). The analysis of data from various data sets reveals that over 90%

of rumen archaea detected in rumen contents belong to one of the genus levels Methanobrevibacter, Methanomicrobium, and large group of uncultured methanogens along with Methanobrevibacter spp (Janssen & Kirs 2008).

In a study conducted by Zhou et al. (2010) it was observed that methanogen populations change in response to diet modifications. The study focused on how methanogen populations in Hereford –Aberdeen Angus steers change in response to diets of low-energy or a high-energy composition and different feed efficiencies. The low energy diet was consistent with bands belonging to *Methanobrevibacter* ruminantium NT7 while the high energy diet had bands consistent with bands belonging to *Methanobrevibacter* smithii*, Methanobrevibacter* sp. AbM4 and *Methanobrevibacter ruminantium* NT7. The low residual feed intake group had a predominant band related to *Methanobrevibacter* sp. AbM4. *Methanobrevibacter* sp. AbM4 and *Methanosphaera* stadtmanae were both represented by sequencing on the methanogenic PCR-DGGE bands. *Methanobrevibacter sp*. AbM4 was also found to be associated with high energy diets and *Methanosphaera stadtmanae* was also associated with low residual feed (Zhou et al., 2010). The study also indicated that the total methanogen population did not differ in response to the different diets or the levels of residual feed intake. The results of the study as with other studies indicated that the predominant species in the ruminal methanogenic population was Methanobrevibacter species. Also various genotypes or strains of the same species were identified and *Methanosphaera stadtmanae* was one of these species. However, there was no difference in species between animals indicating that the change in the methanogenic community must be at the genotype level. The study reported that the impact of the diet on the methanogenic community may be due to substrate utilization, while mutations in the phylotypes may have been the determining factor in what dominant methanogen is present in the rumen.

Therefore, the strain or genotype of the methanogenic population should not be ignored when addressing rumen function (Zhou et al., 2010). This study also gives insight into the fact that the total methanogen population may not be a significant indicator of CH_4 production in the rumen since methanogens differ in substrate preferences and diet can change not just the species but the phylotype present in the rumen. This rumen ecosystem is diet dependent and change in these primary digestors can also impact the survival of methanogens (Zhou et al., 2010).

A previously study (Wright et al., 2004) compared the methanogen population in pasture grazed and forage fed sheep. *Methanobrevibacter* species was the dominant species in grazed and forage fed sheep. It was also reported that *Methanosphaera* stadtmanae was present in pasture grazing sheep and hay fed sheep (Wright et al., 2004). However, a study by (Mohammed, Zhou, Koenig, Beauchemin, & Guan, 2011) showed that diet had no effect on total methanogenic PCR-DGGE bands. The report by (Mohammed et al., 2011) also showed that total copy numbers of total methanogenic 16S rRNA genes did not differ among diets, however it was reported that diet affected the diversity of the methanogens. This further supports the idea that methanogen communities in the rumen may be similar, but diet can affect the species.

Methanogens are not directly involved in digesting fiber, but have been known to enhance the efficiency of the fiber digesting bacteria by preventing the accumulation of reduced nucleotides that inhibit fermentation (McAllister et al., 1996). Ruminal methanogens are sensitive to chemical concentrations in the rumen (McAllister et al., 1996). For example electron acceptors like nitrate and sulfate are known for directing electron flow away from the reduction of CO_2 to CH_4. The reduction of nitrate has a Gibbs free energy value (ΔG) of -163KJ mol $^{-1}$ and sulfate has a ΔG of -152 KJ mol^{-1} and this means that nitrate and sulfate are both thermodynamically favored. This is important because it could support the idea of directing the

electron flow in the rumen away from CO_2 because CO_2 has a ΔG of 130 KJ mol^{-1} and is the least thermodynamically favored of the three reactions (van Zijderveld et al., 2010).

Although methanogens are considered to be at the bottom of the fermentation chain their use of H_2 to reduce CO_2 to CH_4 is an integral part of maintaining efficiency in the host animal by regulating H_2 partial pressures that may inhibit electron transfer reactions (Morgavi, Forano, Martin, & Newbold, 2010). This interaction between methanogens and other microbes is commonly referred to as interspecies hydrogen transfer. However methanogenesis can be affected by the diet and other electron acceptors present in the rumen because other pathways in the rumen utilize H_2 and can be competitive (Morgavi et al., 2010). This competition for H_2 indicates that pH and toxic compounds may have an impact on the composition of the microbial communities in the rumen (Janssen & Kirs, 2008).

It is well known that methanogens are located in various places in the rumen (Janssen & Kirs 2008). The methanogen diversity has been evaluated in rumen fluid, solid, and epithelium from a Korean cow using 16s rDNA sequences in a culture independent technique (Shin et al., 2004). The three fractions of the rumen had similar methanogen families present; however each fraction contained different uncultured groups. *Methanomicrobiaceae* was the dominant group in the epithelium and rumen fluid fractions in the cow whereas *Methanobacteriaceae* was the predominant group in the solid fraction. The study also indicated that the rumen fluid contained the highest proportion (67%) of unidentified clones, followed by rumen solid (40%), and epithelium (5%). These findings suggest that although the rumen is diverse in microorganisms they are selective in terms of location to thrive. Whitford et al. (2011) studied free living methanogens in the bovine rumen of Holstein dairy cows. The two main sequences identified were similar to *Methanobrevibacter ruminantium* (M. ruminantium) and *Methanosphaera*

stadtmanae (M. stadtmanae). However there were groups that represented methanogen species that have not been previously identified by culture techniques (Whitford et al., 2011). The detection of methanogens has led to the identification of Methanobrevibacter as the dominant species in the rumen (Leahy et al., 2010).

1.2.4 Methanogens and Protozoa in the Rumen. Rumen protozoa were first described by Gruby and Delafond in 1843 and this gave some indication that they must be important to the welfare of their host (Morgavi et al., 2010). Fibrolytic microorganisms play an important role in the rumen as they are at the top of the microbial trophic chain as they transform plant cell wall polysaccharides into simple products like VFAs, H_2 and CO_2. The roles of these protozoa are important because they are primary fermenters in the microbial fermentation process. Therefore these protozoa produce large quantities of H_2 which methanogens utilize, which makes their presence vital for the survival of methanogens as they serve as hosts to methanogens and also protect them from O_2, which can be toxic to methanogens (Morgavi et al., 2010). Degradation of plants in the rumen by fibrolytic microorganisms plays a role in methanogenesis as well because they also provide the substrates for methanogenesis.

The association between methanogens and protozoa is of benefit to both species since rumen protozoa depend on H_2 producing fermentation and depend on methanogens for hydrogen removal for optimal conditions for protozoal metabolism. In turn methanogens depend on H_2 for growth and viability. The family *Methanobacteriaceae* was the most abundant species present in the rumen of a Holstein cow (Sharp et al, 1998)which may suggest that this family can exist free living or with protozoa from ruminal fluid collected from a Holstein cow on total mixed ration (TMR) consisting of alfalfa hay, dry shelled corn, soybean meal, dicalcium phosphate, trace minerals, and a vitamin mixture (Sharp, Ziemer, Stern, & Stahl, 1998). This finding indicated

that this family which is comprised of *Methanobrevibacter ruminantium*, *Methanosphaera stadtmanae, Methanobacterium formicicum, Methanobrevibacter smithii* is highly represented in the rumen and there is a preferred relationship between this family and rumen protozoa.

The second most abundant group was Methanomicrobiales (Sharp et al., 1998), but they were not identified in the protozoal fraction of the rumen fluid, which may indicate that they are free living organisms in the rumen fluid. On the other hand, rumen ciliated protozoa are involved in metabolic activity in the rumen by having an influence on fermentation of feed and other microbial populations and their involvement has an impact on fermentation end products including CH_4. These ciliated protozoa in the rumen are not necessarily essential to the animal, but the presence of protozoa influence the production of VFAs in the rumen (Morgavi et al., 2010). Since protozoa produce H_2 and is used by methanogens if you eliminate protozoa by defaunation from the rumen than a shift in CH_4 production.

"The rumen microbial ecosystems are very complex and quantifying these bacteria in response to the presence of protozoa is important for digestion" (Ozutsumi, Tajima, Takenaka, & Itabashi, 2006). Rumen microorganisms are associated with animal productivity and gut health so there has to be an understanding of the correlation between bacteria profiles, diet, and fermentation parameters. This includes dry matter intake and fermentation measurements like VFAs. This was also investigated by (Hernandez-Sanabria et al., 2010) in 58 Hereford-Aberdeen steers on a low roughage diet.

A study focused on analyzing the relationships between protozoa and bacterial populations by quantifying the rumen bacteria in faunated and unfaunated rumens of Holstein cattle (Ozutsumi et al., 2006). It is important to understand the roles of digestion and their relationship between bacteria and protozoa in the rumen. For example amylolytic

bacteria(*Prevotella* species) has been shown to increase in protozoa-free rumens (Ozutsumi et al., 2006) and the absence of protozoa did not seem to affect the amount of cellulolytic bacteria (F. *succinogenes*, R. *albus*, and R. *flavefaciens*) in the rumen. This is important because any change in the microbial population whether it is diet composition or the health of the animal plays a major role in the dynamics of the rumen all the way down to methanogens that are involved in the terminal step in digestion.

Methanogens are thought to be integral components of the microbial food chain in the rumen (Sharp et al., 1998). Methanogens are known to have a symbiotic relationship with ciliate protozoa (Tokura, Ushida, Miyazaki, & Kojima, 1997). Protozoa depend on H_2 forming fermentation and H_2 removal by methanogens is a benefit to protozoa since H_2 inhibits their metabolism. Methanobacteriaceae were the dominant species known to be associated with the protozoal fraction in rumen fluid (Sharp et al., 1998) and *Methanobrevibacter* ruminantium is a member of that family. Other known strains that are a part of this family that have been isolated from similar environments are *Methanosphaera* stadtmanae, *Methanobacterium* formicicum, and methanobrevibacter smithii. Suggesting that there is a preferred relationship with this family and ruminal protozoa.

The protozoa in the rumen that are known to be involved in these relationships belong to the genera Entodinium, Epidinium, Ophryosocolex, and Polyplastron (Hook et al., 2010). The methanogens that are known to be associated with this relationship belongs to the Methanobacteriales, like Methanobrevibacter, and Methanomicrobiales. There has been indication that the location in the rumen has impact on which methanogen species is observed. For example the rumen solid fraction of a Korean cow was known to be predominantly Methanobacteriaceae when compared to the epithelium fraction (Shin et al., 2004), which is the

methanogen commonly associated with the bovine rumen (Hook et al., 2010). Ruminal methanogens can also utilize the products from microorganisms like Ruminococcus spp. and ciliate protozoa through attachment to these organisms in the rumen. The quantity of these methanogens associated with ciliates increased after feeding (Tokura et al., 1997) suggesting the number of methanogens associated with ciliates is regulated by feeding and diet.

In summary rumen ciliated protozoa are involved in metabolic activities in the rumen by having an influence on fermentation of feed and other microbial populations like methanogens. This involvement has an impact on fermentation end products including CH_4.

1.2.4.1 Acetogens in the Rumen. Acetogens are bacteria that produce acetic acid by reducing CO_2 and H_2 and can be an important hydrogen sink in the rumen (Moss et al., 2000). Therefore acetogens can compete with methanogens for substrates in the rumen. There are two types of acetogens that inhabit the rumen: reductive acetogenic bacteria and obligate proton-reducing acetogens that hydrolyze fatty acids (Ellis et al., 2008). Reductive acetogens compete with methanogens for H_2 in the rumen, but this effect on methanogens is minimal due to the fact that acetogens require a higher partial pressure to uptake H_2 than methanogens (Ellis et al., 2008) and are fewer in number in the rumen than methanogens (Moss et al., 2000) making acetogenesis as a strategy to reduce methanogenesis difficult. Methanogens have the ability to reduce H_2 partial pressures and use H_2 as a substrate for methanogenesis (Ellis et al., 2008).

1.2.4.2 Sulfate-reducing Bacteria in the Rumen. Methanogens also have to out-compete other microorganisms for H_2 in the rumen and these sulfate-reducing bacteria are not extensively studied (Ellis et al., 2008). In rumen environments sulfate-reducing bacteria can out-compete methanogens for H_2. These bacteria have a lower H_2 threshold than methanogens that gives them an advantage. However, sulfur containing compounds must be present in high levels in the

rumen to see this effect on methanogens and can explain the reduction of CH_4 by the use of sulfate (van Zijderveld et al., 2010).

1.2.5 Techniques to Detect Rumen Methanogens. The use of culture-based techniques to study the rumen microbial population only accounted for 10-20% of the microbial diversity (McSweeney, Denman, Wright, & Yu, 2007). The DNA-based technologies that allow the use of 16S rDNA analysis have been employed to further understand the complex microbial populations in the rumen (McSweeney et al., 2007). Genomic DNA extracted directly from the rumen should represent the rumen microbial communities when used in studies that focus on the molecular ecology. The methods used to extract DNA from rumen samples include commercial kits (Li et al., 2003; McSweeney et al., 2007) and a repeated bead beating technique (Whitford et al., 2001).

There has been concern over the DNA quality and DNA yields associated with methods to extract DNA used for microbial studies. The quality of DNA and effective cell lysis are crucial when working with sensitive techniques like PCR. A study published by Li et al. (2003) compared the QIAamp® DNA Stool Mini Kit with the bead beating technique to evaluate their effectiveness to examine the gut microbial populations in pigs. Cell lysis in the study was determined by direct counting for both the bead beating technique and the commercial DNA extraction kit with cell lysis efficiencies 96% and 95%, respectively. The study revealed that the % cell lysis, PCR-DGGE profiles, and sequence analysis of random 16S rRNA gene clones that the QIAamp® DNA Mini Stool Kit has efficiency similar to the bead beating technique and is appropriate for ecological studies involving gut microbial populations.

The QIAamp® DNA Mini Stool Kit was also evaluated for its effectiveness for producing high quality DNA for different microbial groups (Henderson et al., (2013). The study

revealed the QIAamp® DNA Mini Stool Kit extracted the highest molecular weight DNA along with two other commercial extraction kits. Also Henderson et al., (2013) indicated that the QIAamp® DNA Mini Stool Kit provides DNA of sufficient quality and quantity compared using quantitative PCR. In relation to archaea DNA the study also revealed that archaea species that are representative in samples for DNA-based techniques depend on the extraction procedure when cell lysis steps are considered (Henderson et al., 2013).

There are DNA-based techniques that can be utilized to describe changes in the rumen microbial populations. Quantitative real-time PCR is a common DNA based technique that allows microbial gene expression to be expressed quantitatively. This method is rapid and estimates expression levels of single and multiple genes. The disadvantage of quantitative real-time PCR is that the technique is based on small subunit ribosomal sequence identities of previously sequenced cultured organisms and clone libraries (McSweeney et al., 2007).

The use of quantitative methods to understand gene expression is valid for molecular biological laboratories (Schmittgen & Livak, 2008). Real-time PCR is a tool used to quantify gene expression. This data from real-time PCR can be presented in absolute quantification by providing exact copy number following transformation of the data using a standard curve or relative quantification where the data is presented relative to another gene as an internal control (Schmittgen & Livak, 2008). The comparative C_T method ($2^{-\Delta\Delta C}{}_T$ method) is a widely used to present real-time data quantitatively (Schmittgen & Livak, 2008). The comparative C_T method makes the assumption that PCR efficiency is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene (Schmittgen& Livak, 2008). It is noted that under this assumption the target and internal gene controls are included in the equation to account for differences in their efficiencies. The data for this method is presented in 'fold change.'

1.2.6 Enteric Methane Production. The accumulation of H_2 can affect the fermentation process and microbial growth so the efficient removal of H_2 is necessary (van Zijderveld et al., 2010). The removal of H_2 from the rumen is important because it directly affects the formation of VFAs and increases fermentation. "The production of H_2 in the rumen is a thermodynamically unfavorable process and in small amounts can inhibit hydrogenase activity in the rumen" (Moss et al., 2000). Although H_2 is one of the major end products of fermentation by protozoa and fungi, it does not accumulate in the rumen because it is continuously used by methanogens present in the mixed microbial ecosystem. This relationship that regulates H_2 accumulation between fermenting species and H_2-utilizing methanogens is called "interspecies hydrogen transfer" (Moss et al., 2000). Even though the bulk of the free-H_2 in the rumen is used by methanogens to produce CH_4 starch-fermenting bacteria can compete for H_2 with methanogens for the production of propionate (Moss et al., 2000).

The rate of CH_4 production is depended on the amount of H_2 introduced into the rumen (Moss et al., 2000) and this metabolic H_2 in the form of reduced protons can be used during the synthesis of VFAs or microbial organic matter.

The amount of CH_4 formed depends on the components of the diet fed to the ruminant animal (Janssen, 2010). When ruminants are fed diets high in fiber they generate a relatively higher amount of CH_4 than when they are fed high grain diets. These findings are because of the less degradable components of plant cell walls in forage type diets and more degradable starches in high grain diets (Janssen, 2010).

1.2.6.1 Methane Production in the Hindgut. Methane production can occur during post-rumen digestion by large amounts of organic matter bypassing the rumen and digestion takes place in the hindgut (Moss et al., 2000). The rumen does not account for all CH_4 that is

produced by the animal (Ellis et al., 2008). This production though small can contribute to the production of CH_4 from an animal. There has been a difference in the amount of CH_4 detected from animals when CH_4 formation in the hindgut was accounted for (Boadi & Wittenberg, 2002). It is also important to note that CH_4 formed during fermentation diffuses through the gut wall into the blood and can be expelled by the lungs (Moss et al, 2000). Also diets that supply ground roughage and maize starch supply organic matter to the hindgut where 10-30% of OM can be digested (Moss et al., 2000). There is evidence that fermentation in the hindgut of animals occurs (Moss et al., 2000; Ellis et al., 2008; Woldeghebriel et al., 2013), but for the measurement of CH_4 production in cattle this discrepancy may not always be accounted for *in vivo* or in models used to predict CH_4 production(Ellis et al., 2008).

1.2.6.2 Variations in Ruminant Methanogenesis. The major source of digestible energy for ruminants is carbohydrates and the type of carbohydrates has been reported to affect CH_4 production (Johnson & Johnson, 1995). Carbohydrates that are readily fermented can lower the rumen pH and shift VFA patterns towards an increase in lactate and propionate (Moss et al., 2000). This can indirectly influence CH_4 by utilizing H_2 for propionate production. Feeding forages that contain cellulose and hemi-cellulose, which degrade slower in the rumen than fermentable carbohydrates increases CH_4 yield (Johnson & Johnson, 1995). It was also reported by Johnson & Johnson (1995) that high grain diets like concentrates vary in gross energy intake (GEI) methane losses and the loss rates fall in between 2 to 3%. The presence of lipids in feed can also reduce CH_4 emissions by decreasing the activity of methanogens (Grainger & Beauchemin, 2011). In other words expressing CH_4 yield in relation to feed intake may impact methanogenesis. The increased intake of concentrate diets from one to two times metabolizable energy (ME) requirements can reduce the percent gross energy (GE) lost as CH_4 by 1.6 in cattle

(Johnson & Johnson, 1995). Variations in CH_4 emissions may occur in animals fed at the same level of intake. Large animal-to-animal variations in CH_4 yield are most likely associated with high intakes, effects of salivation and rumen digestion and passage rate (Pinares-Patino et al., 2007). The variations in CH_4 yields from a lactating and pregnant animal can be observed due to feed intake being maximized unlike a non-lactating animal (Pinares-Patino et al., 2007).

The intake of processed feed that is readily digestible can cause a decline in rumen pH, which can be a consequence for increased VFA production (Janssen, 2010). Grain feeds also lower the pH (Lana et al., 1998), which can impact the optimal pH range for methanogens to grow (McAllister et al., 1996; Lana et al., 1998; Janssen, 2010). This dietary effect on pH may explain the decrease in CH_4 production in ruminants on high grain diets. This is supported by Lana et al. (1998) who reported lower CH_4 production when the rumen pH was less than 6.0. Van Kessel and Russell (1996) also reported that diet can change the rumen pH influencing the utilization of H_2 and thus impacting methanogenesis.

Since methanogens are solely responsible for methanogenesis it is important to acknowledge that other rumen microorganisms are indirectly responsible for methanogenesis because they are involved in H_2 reactions and some share a symbiotic relationship with methanogens (Morgavi et al., 2010). Methanogens attach to feed particles and can affect methanogenesis in the rumen (Morgavi et al., 2010) when the passage rate of digesta is taken into consideration.

1.2.7 Volatile Fatty Acids and Methane Production. Microorganisms in the rumen ferment carbohydrates in the diet to VFAs, which the animal uses as energy sources (Boadi et al., 2004). The VFA equilibrium in the rumen is made possible by cycles in the rumen that mix ruminal contents and VFAs are then exposed and reabsorbed by the rumen epithelium in a

continuous process (Storm & Kristensen, 2010). There are three major VFAs (acetate, butyrate and propionate) that are produced during fermentation and they have different functions in the animal (Boadi et al., 2004). While the two non-glucogenic fatty acids (acetate and butyrate) are used by the animal for milk fat and long-chain fatty acid synthesis (Morvay, Bannink, France, Kebreab, & Dijkstra, 2011) propionate; on the other hand is used for gluconeogenesis. The production of these volatile fatty acids leads to free H_2 to be utilized in the rumen for energy for the animal and other processes (Boadi et al., 2004).

Production of VFA in the rumen is dependent upon the substrate fermented and individual VFAs have different metabolic pathways (Siciliano-Jones & Murphy, 1989; Janssen, 2010; Morvay et al., 2011) and can also depend on the bacterial populations (Dijkstra et al., 2012). Ruminants fed a grain diet produce more propionate in total rumen fermentation products than those fed a forage diet (Van Kessel & Russell, 1996; Lana, Russell, & Van Amburgh, 1998; Russell, 1998; Janssen, 2010). The propionate pathway has been suggested to be an alternative for accepting electrons or H_2 formation. The increase in propionate production has been correlated with the decrease in CH_4 production. This is supported by the fact that the propionate pathway consists of pyruvate being converted into propionate. The addition of cereal grains to the diets of ruminant animals has been known to cause a decrease in methane production and an increase in propionate production. There has also been interest in methanogens and their ability to maintain low partial pressures of H_2 and allow other microbial species to produce VFA end products (Dijkstra et al., 2012).

A study by Van Kessel and Russell, 1996 indicated that the forage diet (consisting of timothy hay) pH was constant between 6.7-6.9 and acetate was the dominant fermentation acid, and the acetate: propionate ratio was 4.2 (Van Kessel & Russell, 1996). The study indicated the

cereal grain (corn and soybean meal-concentrate diet) lowered the pH and the total VFA concentration increased. The acetate: propionate ratio was 1.8. In addition to adding H_2 gas to the concentrate diet samples there still was little to no production of CH_4 while the forage diet increased the production of CH_4. Volatile fatty acid concentrations have been shown to increase linearly when Holstein steers were fed higher amounts of concentrate and decreasing amounts of forage (Lana et al., 1998) and the acetate to propionate ratio decreased as well as the ruminal pH. (Russell, 1998) reported that fermentation end products like VFAs are diet dependent and cereal grains generally have lower acetate: propionate ratios (Russell, 1998). For instance cows fed 90 % concentrate had lower acetate: propionate ratios by an increase in propionate production and increased total VFA concentrations than forage fed cows (Russell, 1998).

There is a relationship between the acetate and propionate ratio and CH_4 formation in the rumen (Boadi et al., 2004) as shown in the equations below. The three major VFA pathways are as follows:

Equation 1 $C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2$ *(Acetate)* $+ 2CO_2 + 8H \rightarrow$

$(CO_2 + 8H \rightarrow CH_4 + 2H_2O)$

Equation 2 $C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2$ *(Propionate)* $+ 2H_2O$

Equation 3 $C_6H_{12}O_6 \rightarrow C_4H_8O_2$ *(Butyrate)* $+ 2CO_2 + 4H \rightarrow$

$(CO_2 + 4H \rightarrow CH_4 + 2H_2O)$

Equations 1-3: Illustrate the role of the type of VFAs in CH_4 production (Boadi et al., 2004)

"It has been known for many years that changes in the molar proportions of the concentrations of VFA in the rumen can be induced through a wide variety of dietary manipulations" (Sutton et al., 2003, p.3620). The study measured the production of VFA in 5

Friesian mid-lactation cows on regular and low roughage (LR) diets with concentrate that consisted of rolled barley and extracted soybean meal with a high starch (low fiber) content (Sutton et al., 2003). The hay used in the study was mature with high fiber concentrations. It was reported the LR diet had a lower pH and higher molar proportions of acetate than LR, while the LR diet had higher molar proportions of propionate and a significantly lower acetic and butyric acid concentrations. The study indicated net production of VFA was affected by diet and net energy was also affected by the introduction of a low roughage diet, while propionic acid doubled and net energy from total VFA production increased.

1.2.8 Effect of pH in Methane Production, Fermentation, and VFA Profiles. The rumen environment plays a major role in enteric CH_4 production. The rumen provides its microorganisms with an environment that is suitable for growth and in turn the ruminant benefits from digestion processes that provides VFAs for energy. It has been noted that diet can play a major role in altering the pH in the rumen and having an impact on methanogenesis and other fermentation end products like VFAs (Lana et al., 1998; Russell 1998; Van Kessel & Russell 1996). The effect rumen liquid volume, pH, and concentrations of VFA on the rates of absorption of acetic, propionic, and butyric acids from the rumen was evaluated in lactating crossbred Friesian/Holstein-Friesian cows (Dijkstra, Boer, Van Bruchem, Bruining, & Tamminga, 1993). It was noted that a decrease in pH increased fractional absorption rates of VFAs (Dijkstra et al., 1993). Also the accumulation of VFAs in the rumen can reduce ruminal pH (Dijkstra et al., 2012). In return the pH of the rumen can cause a shift in favor of certain bacteria that may impact fermentation and influence CH_4 production. Methane production has also been shown to decrease as the pH decreases below 6.5 (Van Kessel & Russell, 1996).

The pH of the rumen has been shown to have a correlation with *in vitro* methanogenesis. The relationship between pH and ruminal methanogenesis was investigated in Holstein cows fed two different diets consisting of forage (Timothy hay) and concentrate (corn and soybean meal; Van Kessel & Russell, 1996). The forage diet had a pH range between 6.7 and 6.9 while the concentrate diet had a pH range between 5.4 and 6.1. The forage diet had higher amounts of acetate and lower amounts of propionate and butyrate concentrations than the concentrate diet. There was also an increase in total VFA from the concentrate diet. (Van Kessel & Russell, 1996) indicated that the forage diet had no significant decreases in rumen pH and higher amounts of CH_4 was detected while the concentrate diet showed a decrease in rumen pH and little or no CH_4 was detected. This suggests that methanogens may be pH dependent. This relationship between pH and CH_4 is important to understand because this provides insight into the effect that pH has on CH_4 production. Some credit has been given to starch and fiber digesting bacteria for their role in the production of fermentation end products like VFAs (Russell, 1998). Methanogens are sensitive to changes in the rumen environment, so pH may inhibit their ability to use reducing equivalents like H_2 to produce methane from digestion. This is supported by Russell, 1998 who reported that at a pH lower than 6.0 hay fermentation was inhibited, which would be expected if the assumption is that ruminal celluloytic bacteria fail to adapt to low pH (Russell, 1998). This would impact methanogens that share a symbiotic relationship with these bacteria.

Rumen pH and microbial populations can be influenced by the type of carbohydrate in animal feed, which in turn impacts the amount of CH_4 produced during fermentation. Therefore, (Johnson & Johnson, 1995) performed a regression analysis of data retrieved from the literature on beef cattle that indicates fermentation of plant cell walls leads to higher methane production

and higher acetic to propionic acid ratios. The study also indicated that non-plant cell wall components of feed like sugars and starch vary in their methanogenic capacity.

Lana et al. (1998) indicated that steers that were fed a diet consisting mainly of concentrate had an increase in total VFAs, but a decrease in pH, ammonia, and acetate to propionate ratios. Therefore it is well established that if the rumen pH is lowered there should be a decrease in the acetate to propionate ratio, which will lead to a decrease in CH_4 and NH_3 production and a possible increase in propionate production. Ammonia production correlated with rumen pH and NH_3 production decreased as *in vitro* pH decreased in the rumen from cattle fed forage based diet (Lana et al., 1998).

It is well known that lower ruminal pH can affect microbial populations, which is linked to decreased digestion in the rumen (Dijkstra et al., 2012). Since there is a link between rumen fermentors and pH in the rumen it is important to consider how this impacts the availability of substrates in the rumen. Diets that result in high VFA concentrations can lead to a decline in fiber digestion because of low pH and lower the effectiveness of nutrient absorption by the animal (Dijkstra et al., 2012). Cellulolytic bacteria are also known to be affected by low pH which impacts cellulose activity (Dijkstra et al., 2012). The change in pH due to diet whether it is adapting to a new feed or feed additive is important because the change in bacterial communities, fiber digestion and VFA concentrations all play a role in the rumen environment and eventually enteric CH_4 formation.

1.2.9 Particle Size and its Effect on Fermentation Products in the Rumen. Forage processing could impact the rate of passage of ingested feed given indicating that reducing the particle size could influence CH_4 emission from cattle (Johnson & Johnson, 1995). Johnson & Johnson (1995) indicated that a 28% of variation in CH_4 production in the literature was related

to passage rate of the feed. It is also known that a reduction in feed particle size (PS) can increase microbial attachment which can increase digestion and VFA production (Bhandari, Li, Ominski, Wittenberg, & Plaizier, 2008). The effect of corn silage particle size on dairy animals in relation to fermentation is very important. The fiber content of feed is known to stimulate chewing activities. Also mastication reduces particle size of ingested feed and is necessary for the consumption of feed (Kononoff, Heinrichs, & Lehman, 2003). This primary process in digestion should have an impact on rumen function if the feed was manipulated before even being fed to the animal. It is also well known that ruminating time is increased by longer particle sizes of feed. Also the rapid removal of feed from the rumen could result in lower pH (Kononoff et al., 2003). The particle size of the feed may affect the rumen environment by having an impact on the fermenting bacteria that inhabit the rumen.

Kononoff et al. (2003) evaluated the effect of different particle sizes of corn silage *in vivo* on chewing activities and rumen fermentation in lactating Holstein cows. The animals were offered TMR that was chemically similar, but differed by particle sizes as short (SH), mostly short (MSH), mostly long (MLG), and long (LG). The study reported a linear increase in DMI and NDF intake as the particle size decreased. Also the effect of particle size on DMI was greater when the SH and LG were compared. The study indicated the eating and ruminating time decreased as particle size decreased. The concentration of total VFA had also increased linearly as particle size of the silage decreased. There was no effect observed on rumen pH and NH_3 concentrations, although this effect did not coincide with the increased concentrations of VFA observed in relation to particle size (Kononoff et al., 2003). The intermediate particle sizes had the greatest effect on acetate and propionate where molar acetate increased and propionate decreased. This result could be due to starch and fiber intake (Kononoff et al., 2003). This study

indicates that the smaller particle size of silage increased fiber intake, which in turn stimulated chewing activity. The shift in VFA production shows that acetate and the acetate to propionate ratio exhibited a quadratic effect with MSH and MLG having the highest molar concentration whereas propionate had a slight decrease with the smaller particle size.

The effect of reducing alfalfa haylage particle size (PS) on Holstein cows in early lactation was evaluated by Kononoff & Heinrichs, (2003). The PS for the study was considered long (22.3 mm) and short (4.8 mm) forage. There was no chemical difference reported for the feed used in the study. The study did indicate that total VFAs, acetate, butyrate, and propionate increased and the acetate to propionate ratio decreased with reducing PS, while there was no effect on ammonia reported. It was observed by Kononoff & Heinrichs, (2003) that animals that consumed the short PS also had increased digestibility and a lower pH than animals that did not consume the short rations. The study concluded that these results indicated there was an increase in the surface area for microbial attack and the availability of substrates for rumen microbes with the reduction in PS.

A study conducted by Krause & Combs, (2003) investigated the effects of forage PS on DMI, performance, digestibility, microbial yield, ruminal pH, and chewing activity in Holstein cows. It was reported there was a decrease in DMI and an increase in milk production with a decrease in forage PS. It was also reported that there was an increase in microbial protein as forage PS decreased. The concentration of propionate and the acetate to propionate ratio decreased as forage PS decreased, however when it was expressed as molar percentages acetate decreased and there was an increase in propionate. It was also reported by Krause & Combs, (2003) that mean ruminal pH was not affected by forage PS.

The effect of forage chop length on pH and rumen fermentation has been evaluated on sixteen lactating Holstein cows (Bhandari et al., 2008). The study concluded that reducing the chop length of alfalfa and oat forage increased in DMI for the shorter length of oats. The daily rumen pH, rumen liquid outflow, and the concentrations of VFA in the study were not affected by the chop lengths of both forages. Also there was no effect on the acetate to propionate ratio and NH_3 concentration in the rumen was not affected by the different chop lengths of forage. Although the particle size of feed is important in the aspect of animal health and rumen health for example, DMI and rumen fermentation, the cascade of events from manipulation, mastication, fermentation, and the end products can be related to formation of enteric CH_4.

The forage particle size and concentrate level has been evaluated for the impact on fermentation profiles in particle associated rumen fluid and free rumen liquid from late lactation Holstein cows (Zebeli, Tafaj, Weber, Steingass, & Drochner, 2008). Diet composition consisted of low concentrate with fine chopped hay, low concentrate with long chopped hay, high concentrate with fine chopped hay, and high concentrate with long chopped hay. The PS of the grass hay was 6mm and 30mm. The PS is one of the factors that determine the structural fiber in the diet of dairy cows (Zebeli et al., 2008). "Ruminal contents are very heterogeneous physically and microbiologically" (Zebeli et al., 2008). Cellulolytic bacteria are known to be associated with particulate containing rumen fluid whereas free rumen fluid contains microorganisms not associated with particles. This assumption which gives some indication that the effect of PS will vary on rumen fermentation depending on the location in the rumen. The particulate rumen fluid had higher VFA concentrations and lower pH than the free rumen fluid (6.23 and 6.52 respectively). Dietary forage PS did not affect gas production. The sample site did have an effect on *in vitro* parameters. Rumen fluid associated with particulate matter produced more gas

than free rumen fluid and the concentrate diet levels used in the study effected the fermentation profiles the most (Zebeli et al., 2008).

The effect of PS and DM content was evaluated in Danish Holstein cows (Storm & Kristensen, 2010). The grass hay had a PS size of 3.0 and 30 mm in length. The PS size is known to affect the size of the ingested material. Also PS has been shown to effect ruminal pH. (Storm & Kristensen, 2010) reported DM did differ between the two PS. It was also reported by (Storm & Kristensen, 2010) that chewing time was longer for the longer PS when compared to the shorter PS and eating time was not associated with PS. The study noted rumination time increased for the longer PS when compared to the smaller PS. There was no PS effect on milk fat concentration and previously digested feed VFA and ruminal pH (Storm & Kristensen, 2010). Teimouri Yansari et al. (2004) investigated the effect of particle size from corn silage based rations in mid-lactation Holstein cows. It was reported that the chemical composition of the feed was not affected by the reduction of particle size. The study also reported a decrease in ruminal pH with the reduction in PS, which corresponded to an increase in propionate and lower acetate to propionate ratio. It was also noted in the study that particle size had no effect on ammonia concentrations. The study conducted by Teimouri Yansari et al. (2004) concluded that feed particle size had an effect on fermentation namely by increasing DMI, ruminal particle passage rates, total VFAs, and propionate individually. However it was also reported that there was a decrease in ruminal mean retention time, acetate to propionate ratio, and rumen pH.

1.3 The Use of Nitrate and Fumarate as Feed Additives

1.3.1 Fumarate as a Feed Additive. Fumarate is a dicarboxylic acid intermediate product in tricarboxylic acid cycle. Dicarboxylic acids including fumarate have long been proposed as rumen modifiers by enhancing the succinate-propionate pathway (Araújo, Nunes-

Nesi, & Fernie, 2011). Fumarate is a key intermediate in rumen microbial metabolism (Yu et al., 2010). Fumarate acts as a rumen buffer by increasing CO_2 production and improving lactic acid utilization (Castillo et al., 2004). Several investigators (Bayaru et al., 2000; Carro & Ranilla, 2003; Castillo et al., 2004; Mao et al., 2008; Abdl-Rahman, Sawiress, & Abd El-Aty, 2010; Wood et al., 2009; Yu et al., 2010) have used fumarate as a feed additive to alter rumen fermentation and act as an electron acceptor and reduce methanogenesis but increase VFA production. The metabolic fate of fumarate is shown in Figure 2. It takes one mole of fumarate to seize one mole of H_2 away from methanogenesis, which can then be used to reduce succinate and eventually to propionate (Ungerfeld et al., 2007: Figure 2).

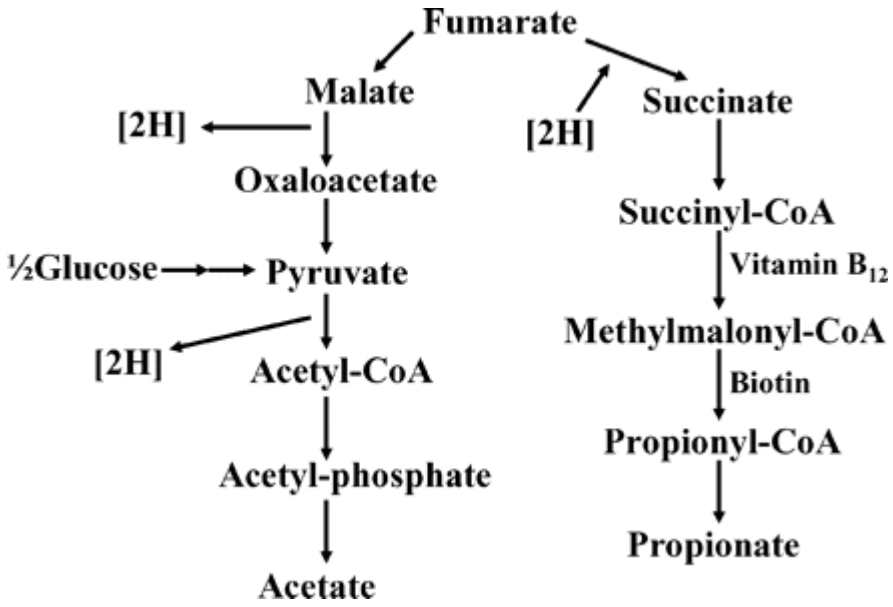

Figure 2. The Metabolic Fate of Fumarate.

It is estimated that the reduction of fumarate in the rumen should be favored over methanogenesis due to Gibbs free energy that is released per pair of electrons incorporated into fumarate reduction and methanogenesis making reduction of fumarate more exergonic (Ungerfeld et al., 2007). The addition of the appropriate amount of fumarate in the diet could compete for H_2 and reduce CH_4 production in the rumen (Ungerfeld et al., 2007). Fumarate

decreases methanogenesis by competing for H_2 with methanogens and it is expected that there is a stoichiometrical relationship between a decrease in CH_4 and fumarate added to a system (Ungerfeld et al., 2007).

In vitro studies (Carro & Ranilla, 2003; Abdl-Rahman et al., 2010; Lopez, Valdes, Newbold, & Wallace, 1999; Castro-Montoya, De Campeneere, Van Ranst, & Fievez, 2012) varying in results for the effect of fumarate on CH_4 production indicate a difference between adding fumarate continuously or as a one–time dose (Wood et al., 2009). The presence of fumarate reducing bacteria in the rumen increased when fumarate was added to the ruminant diet (Asanuma, Iwamoto, & Hino, 1999). In the bacterial community these fumarate reducing bacteria are low in numbers and are slow growing (Asanuma et al., 1999). While H_2 is the primary substrate for methanogenesis, they can also use fumarate as a substrate. Formate is usually converted to CO_2 and H_2 before being reduced to CH_4. Fumarate utilizing bacteria have a greater affinity for formate, which is a substrate utilized by methanogens to produce CH_4 (Asanuma et al., 1999). The low efficacy of fumarate may be associated with the pathway of converting fumarate to propionate and the conversion of fumarate to acetate (Ungerfeld et al., 2007). The role of organic acids in methanogenesis in general could be beneficial by directing H_2 and formate, which are the major substrates for CH_4 production to be utilized by other non-methanogenic bacteria and to reduce CH_4 production (Castillo et al., 2004).

Fumarate has also been observed to enhance rumen fermentation by increasing propionate production and depending on the animal's diet it can provide optimal conditions for cellulolytic bacteria to grow (Castillo et al., 2004). The modification of the rumen by fumarate results in a shift of CH_4 production through the production of propionate, which would then lead

to less free H_2 that could have reduced CO_2 to CH_4. This process can be beneficial to the animal by increasing production of propionate and reducing CH_4 emission.

According to Asanuma et al. (1999) and Lopez et al. (1999) the addition of 20mM of fumarate to batch cultures incubated for six hours decreased CH_4 formation by 5% and increased propionate production by 56%. The addition of 30mM of fumarate also revealed an 11% decrease in CH_4 and a 58% increase in propionate proportions compared to the control samples. These studies indicate that fumarate was metabolized to propionate. However higher concentrations of fumarate produced a higher proportion of succinate, but after a longer incubation period sunccinate itself was metabolized to propionate (Lopez et al., 1999).

Previous studies (Abdl-Rahman et al., 2010; Wood et al., 2009; Yu et al., 2010; van Zijderveld et al., 2011; Castro-Montoya et al., 2012) have set the direction for further research to identifying fumarate as a possible mitigation strategy for methanogenesis by using H_2 to produce propionate and reduce H_2 availability for CH_4 production in the rumen. However, Beauchemin and McGinn (2006b) indicated that fumarate had no effect on total daily CH_4 emissions *in vivo*. An *in vitro* study by Mohammed et al. (2004) revealed fumarate reduced CH_4 production and increased propionate production. It was reported that one of the limitations in using dicarboxylic acids to decrease enteric CH_4 production is it must be administered daily which makes it less practical for grazing animals (Mohammed et al., 2004).

The effects of fumarate on *in vitro* ammonia (NH_3) concentration and fiber digestion using goats as rumen fluid donors was investigated (Yu et al., 2010). The batch cultures of mixed rumen microorganisms were combined with sources of nitrogen (N) as casein amino acids, ammonium bicarbonate and others to show the role of fumarate in N utilization. The addition of fumarate can be beneficial for microorganisms providing energy and carbon skeletons for the

utilization of NH_3 (Yu et al., 2010). Fiber degrading bacteria are also enhanced by the addition of fumarate and as a result fiber digestion improves (Yu et al., 2010). However diets high in forage can lead to high CH_4 concentrations (Yu et al., 2010). Also animals fed low quality forage or concentrate can have an imbalance in protein and carbohydrate fermentation, which can lead to high NH_3 accumulations in the rumen (Yu et al., 2010). It may be beneficial to add fumarate which can decrease NH_3 concentrations and increase propionate production, and possibly reduce methanogenesis. Since fumarate has been shown to positively impact rumen fermentation this feed additive could be a good candidate for the reduction of CH_4 and improve the availability of substrates needed for glucogenesis (Yu et al., 2010).

The addition of fumarate to batch cultures decreased ruminal NH_3 accumulation linearly as the dose of fumarate increased while the addition of fumarate increased total VFA concentrations and reduced the acetate to propionate ratio. It was also observed (Yu et al., 2010) that the addition of fumarate enhanced the utilization of NH_3 by rumen micro-organisms in the presence of added nitrogen sources. This effect suggests that fumarate provides a key metabolic intermediate to improve N utilization (Yu et al., 2010). There was no effect on pH reported for the study (Yu et al., 2010); however others (Carro & Ranilla 2003; Lopez et al., 1999; Mao et al., 2008) have reported that fumarate can increase *in vitro* and *in vivo* rumen pH. In the study by Yu et al. (2010) fumarate also increased digestibility and crude protein utilization. These results give way to understanding how fumarate may play a role in rumen fermentation that could be beneficial to reducing CH_4 production.

To minimize the decrease in pH from use of the free acid Carro and Ranilla (2003) evaluated the effect of disodium fumarate with different concentrates feeds, primarily cereal grains, on *in vitro* CH_4 production in sheep. The study revealed that in the presence of all

concentrate feeds the pH was increased linearly, which contradicts with the report of Yu et al. (2010) and (Bayaru et al. (2000) who reported of no effect on pH. Also CH_4 was decreased linearly in the presence of all concentrates with the addition of fumarate (Carro & Ranilla, 2003). Total VFA concentrations were increased in the concentrate feeds when fumarate was added. The acetate to propionate ratio decreased while there was an increase in propionate and acetate. The study reported no treatment effect on butyrate. In the absence of all the concentrate feeds fumarate increased ruminal pH and total gas production, but CH_4 production was constant. This may suggest the impact of fumarate on methanogenesis *in vivo* is diet dependent (Carro & Ranilla, 2003). The implications of this study support that fumarate is beneficial in animals on concentrate feeds due to the increase in pH which can counteract the low rumen pH observed from these diets. Fumarate also was observed to increase acetate and propionate, while reducing CH_4 suggesting fumarate effectiveness without suppressing fermentation (Carro & Ranilla, 2003).

The use of fumarate was also supported by Castro-Montoya et al. (2012) who evaluated fumaric acid on three substrates grass silage, maize silage, and concentrate diets on *in vitro* CH_4 and total VFA production in dairy cattle. Fumaric acid decreased CH_4 by 25-50% and increased VFA production when added to all diets. When CH_4 was expressed relative to VFA concentration the inhibition of fumaric acid was greatest in the concentrate sample compared to the grass silage sample.

Disodium fumarate has also been evaluated for its effect on rumen bacterial communities and ruminal metabolism in goats (Mao, Zhang, & Zhu, 2008). The basal diet consisted of alfalfa hay, corn, and soybean meals. The study resulted in a linear increase in pH with increases in fumarate. However, there was no effect on total VFA and NH_3 concentrations with the increases

in disodium fumarate. Disodium fumarate reduced lactate acid and increased ruminal pH while increasing diversity of the rumen bacterial community (Mao et al., 2008). This study suggests that fumarate utilization by microorganisms is influenced by diets (Mao et al., 2008).

The addition of fumarate to ruminant diets has been evaluated *in vitro* using goats as ruminal donors (Asanuma et al., 1999).This experiment suggested that fumarate reduces methanogenesis and enhances propionate production. The addition of fumarate *in vitro* indicated a reduction in CH_4 and an increase in propionate production on cultures fermenting hay powder and concentrate diets. The use of fumarate may be successful at reducing methanogenesis and increasing propionate production in the rumen under favorable conditions that stimulate fumarate-utilizing bacteria which can compete for H_2 with methanogens (Asanuma et al., 1999).

The effect of fumarate on ruminal pH indicates that there could be a decrease in rumen pH. This decrease in pH could limit the amount of fumarate that can be fed to ruminants meaning that "the use of salts of fumaric acid should be employed instead", although the use of salts in ruminant diets should be avoided because of its osmotic load (Wood et al., 2009). Encapsulated fumaric acid has been evaluated in lambs for its effect on performance and CH_4 production (Wood et al., 2009).The addition of encapsulated fumaric acid did not show any decrease in rumen pH. The report indicated that there was an 11% and 19% decrease in CH_4 production from fumaric acid and encapsulated fumaric acid respectively. Encapsulated fumaric acid with coconut oil and palm oil also showed a decrease in CH_4 production by 12 and 20% respectively (Wood et al., 2009). However, the oils alone had no effect on CH_4 and propionate production. Both fumaric acid and encapsulated fumaric acid showed an increase in propionate production. The results of the study suggest that encapsulated fumaric acid may be a possible strategy to inhibit CH_4 formation in the rumen due to its slow release of fumaric acid which

decreased the production of CH_4 and prevented the protozoa from adapting quickly to the addition of fumarate (Wood et al., 2009).

Since previous research (Asanuma et al., 1999) has indicated that fumarate reducing bacteria compete with methanogens for free H_2, fumarate is considered to be a desirable propionate precursor and electron acceptor, but its efficacy *in vitro* and *in vivo* varies. Therefore, the use of this additive may be of benefit when coupled with another feed additive for supplementation due to conditions inside the rumen (Abdl-Rahman et al., 2010).

The effect of fumarate coupled with sodium-lauryl sulphate (SLS) has also been evaluated *in vitro* in steers (Abdl-Rahman et al., 2010). Sodium lauryl sulfate coupled with fumaric acid on methanogenesis showed that gas production was not affected by the addition of fumaric acid alone (Abdl-Rahman et al., 2010). Total gas production was decreased by the SLS-fumaric acid combination, but not by fumaric acid alone. The greatest reduction in CO_2 and CH_4 resulted from the use of SLS-fumaric acid treatment and fumaric acid had no effect on CO_2 but a slight effect on CH_4 production. All treatments seem to decrease the pH. SLS-fumaric acid decreased ammonia and VFA concentrations. Fumaric acid alone did not alter total VFA concentrations, but did increase propionate concentrations along with SLS-fumaric acid combination. The study also revealed fumaric acid and SLS-fumaric acid lowered the acetate to propionate ratio. The use of SLS-fumaric acid combination did not alter the activity of cellulolytic bacteria and fiber digestion.

Calcium fumarate was evaluated (van Zijderveld, Fonken, et al., 2011) along with lauric acid, myristic acid, and linseed oil in lactating dairy cows fed TMR (van Zijderveld, Fonken, et al., 2011). The study reported a 10% decrease in CH_4 production when diet containing calcium fumarate, lauric acid, myristic acid, and linseed oil were fed. The study also observed a decrease

in energy loss in the form of CH_4, an increase in propionate and a decrease in acetate and butyrate. The study by also indicated the combination of calcium fumarate, lauric acid, myristic acid, and linseed oil decreased CH_4 production in lactating dairy cows, but the decrease in CH_4 production was due to the CH_4 suppressing medium chain fatty acids and not due to the H_2 sink potential of calcium fumarate.

The impact of several feed additives (fumaric acid, essential oil, canola oil, spice extract) that are registered for use in feeding cattle were evaluated *in vivo* for their impact on enteric methane formation (Beauchemin & McGinn, 2006b) using 16 Angus heifers on a high-forage diet. The heifers received 175g/day of fumaric acid and 75g/day of sodium bicarbonate to help neutralize the acidity from adding fumarate to the diet. Fumaric acid changed the VFA profiles by slightly decreasing acetate and butyrate. However, there was a slight increase in propionate compared to the control diet. It was also reported that fumaric acid had no effect on total daily CH_4 emission compared to the control. Since the study was evaluated *in vivo* the production and absorption of VFA concentrations in the rumen should be considered. Beauchemin and McGinn (2006a) suggested that the increase in VFA concentrations from the addition of fumarate may have been due to increases in rumen digestion.

Silage based diets in the U.S. are common for cattle and it is important to evaluate feed additives in combination with common diets fed to ruminants. The effect of fumaric acid on *in vivo* CH_4 production, ruminal fermentation, and digestibility has been investigated (Bayaru et al., 2000) in Holstein steers that were fed a silage based diet. The total number of protozoa was not affected by the addition of fumarate. Also fumarate reduced ammonia-N concentrations suggesting a possible increase in N utilization. On the other hand, Bayaru et al. (2000) observed *in vivo* CH_4 production by 23% decrease in diets supplemented with fumaric acid.

The effect of fumarate on CH_4 production has been analyzed on *in vitro* batch cultures and in semi-continuous rumen simulation technique (Rusitec) using rumen fluid from sheep (Lopez et al., 1999). The report from the study indicated that the pH and total gas production was increased while CH_4 production decreased as the amount of fumarate increased. The individual VFAs propionate and acetate were also increased by the addition of fumarate. However, in the Rusitec method total gas production was not affected but CH_4 production decreased. The addition of fumarate also increased the number of cellulolytic bacteria along with digestion. Although the use of fumarate inhibited CH_4 production to some degree it was still a small percentage. These findings suggest that the use of fumarate alone may not be effective for *in vivo* use as the primary method to reduce CH_4 production (Lopez et al., 1999).

1.3.2 Nitrate and Enteric Methane Production. Nitrate (NO_3) is an inorganic salt of a nitrogen oxide that is present in the diets of ruminants (Anderson & Rasmussen, 1998). One of the nutrient requirements of ruminants is amino acids and the microorganisms in the rumen can partially meet this requirement (Leng & Nolan, 1984). Nitrate can be considered non-protein source of nitrogen and can be replaced by urea in the ruminant diet (van Zijderveld et al., 2011). Nitrate is rapidly converted to ammonia in the rumen (equation 4).

Equation 4 $NO_3^- \longrightarrow NO_2^- \rightarrow NO \rightarrow (NOH) \rightarrow NH_2OH \rightarrow NH_4^+$

$\searrow N_2$

Proposed scheme of nitrate reduction (Farra & Satter, 1971)

Nitrate has a high redox potential and is capable of reducing CH_4 production from microorganisms in the rumen (Bozic et al., 2009; Sar et al., 2004; Zhou et al., 2011). The reduction of nitrate is energetically more favorable than the reduction of CO_2 to CH_4.

One of the effective methods for reducing enteric CH_4 production in ruminants would be to reduce the availability of H_2 or provide an electron sink in the rumen. The alternative electron acceptor pathway must be more favorable than the reduction of CO_2 to CH_4 (Hulshof et al., 2012). *In vivo* studies (van Zijderveld et al., 2010; Hulshof et al., 2012) have also indicated that nitrate is effective at reducing CH_4 emission without any adverse effect on rumen fermentation (Bozic et al., 2009). The reduction of NO_3 to NH_3 produces nitrite (NO_2) a toxic compound that can result in methemoglobinemia, but the activity of nitrate reducing organisms can protect against toxic nitrogen compounds (Anderson & Rasmussen, 1998). Feeding practices such as allowing the animal to gradually adapt to the nitrate in the diet has been proven to decrease the risk of methemoglobinemia (van Zijderveld et al., 2010). It has been reported by Zhou, Yu, et al. (2011) that sodium nitrate reduced *in vitro* CH_4 and total VFA production in ruminal fluid collected from a jersey bull fed rye grass (Zhou, Meng, & Yu, 2011). It was also reported (Zhou, Yu, et al., 2011) that sodium nitrate increased the acetate to propionate ratio and reduced methanogens and the number of cellulolytic bacteria.

Sodium and potassium nitrate were incorporated into high grain ration and fed to a non-lactating jersey cow up to 6% of total feed intake and as the amount of NO_3 increased rumen acetate concentration increased while propionate and butyrate decreased (Farra & Satter, 1971). It was also noted that feed intake was reduced when NO_3 exceeded 4% of dietary intake. However, in cows fed high grain diet, NO_3 at 2% of intake increased acetate, decreased propionate with no effect on butyrate. Addition of NO_3 also increased rumen ammonia concentration while milk composition was unaffected. The study indicated that there was no consistent relationship between *in vitro* VFA production and dietary NO_3. Nitrate reduction was more rapid (74%) in ingesta samples that was adapted to the addition of nitrate than ingesta

samples not adapted to nitrate (23.1%). It was concluded by the study that NO_3 can serve as an alternate electron acceptor in rumen fermentation (Farra & Satter, 1971).

Sodium nitrate has been studied *in vitro* with a Jersey bull as the rumen fluid donor for its impact on methanogens and cellulolytic bacteria populations and CH_4 production (Zhou et al., 2011). Methane production was decreased by 70% with the addition of sodium nitrate and as the dose increased CH_3 was nearly completely inhibited. The pH was also increased as the amount of NO_3 increased suggesting increased NH_3 production. On the other hand, total VFAs decreased as the dose of NO_3 increased. Acetate increased at the lower NO_3 concentrations while it was not affected at the highest concentrations and as the concentration of nitrate increased the acetate to propionate ratio increased linearly. However propionate decreased with the addition of NO_3.The implication of this study seems to suggest that nitrate uses H_2 for its own reduction.

The effect of NO_3 was compared with nitroethane and lauric acid with marine algae on *in vitro* CH_4 production using rumen fluid from a Holstein-Friesian cow fed rye-grass (Bozic et al., 2009). The report indicated that CH_4 and total gas productions were reduced, acetate was increased and propionate decreased by the addition of nitrates, which was in full agreement with the most recent report by Hulshof et al. (2012) confirming that NO_3 competes for H_2 with two major pathways (methanogenesis and propionogenesis). There was also an increase in the acetate to propionate ratio and NH_3-N concentrations by the addition of NO_3.

The potential and gradual adaptation of rumen microbes to NO_3 was also examined (Zhou et al., 2011) and the in vivo/in vitro results indicated that the number of the methanogen populations was reduced by 97%. The F. succinogenes, R. albus, and R. flavefaciens populations were also reduced by the addition of NO_3 suggesting that they were sensitive to NO_3

or the inhibition of reducing substrates (Zhou et al., 2011). The high redox potential of NO_3 may have inhibited the growth and metabolism of these species (Zhou et al., 2011). Some cellulolytic bacteria managed to adapt to the NO_3, however methanogens did not seem to adapt to NO_3 or the reduction intermediates like nitrite proving that NO_3 even at low concentration can affect the methanogen populations.

The mechanisms by which NO_3 reduces CH_4 was believed to be due to the direct impact on methanogens (Zhou et al., 2011), however according to Anderson & Rasmussen (1998) there may not be a direct impact on the methanogen population. The effect of nitrate and 3-nitropropionate with mixed ruminal populations was evaluated with the rumen bacteria strain NPOH1 that uses energy from the reduction of nitrocompounds and divert reducing equivalents away from methanogenesis. Methane production was inhibited by 3-nitropropionate and was dose-dependent, however when NPOH1 was added the amount of 3-nitropropionate metabolized increased, but did not affect CH_4 production (Anderson & Rasmussen, 1998). From culture studies 3-nitropropionate was confirmed to be toxic to *Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* inhibiting their growth (Anderson & Rasmussen, 1998). The addition of NPOH1 reduced CH_4 production while the rate of disappearance of NO_3 was increased with NPOH1. This indicates that free H_2 or electrons were diverted away from CH_4 production and used for NO_3 reduction. However there was no evidence that NO_3 was toxic to methanogens in culture studies (Anderson & Rasmussen, 1998), but increasing levels of NO_3 decreased CH_4 production. Therefore the study concluded that NO_3 and 3-nitropropionate reduces CH_4 via two different mechanisms with NO_3 directing reductants away from methanogenesis for its own reduction to NH_3.

The known toxicity of NO_3 reduction into nitrite has led to the investigation of nitrate reducing bacteria in the presence of NO_3 in mixed ruminal cultures (Miwa Iwamoto, Asanuma, & Hino, 2002). The rates of NO_3 reduction, tolerance of bacteria to nitrite, and the effect of these bacteria on rumen CH_4 concentration was investigated *in vitro* in goats on a high roughage diet. *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogenes* are known to reduce nitrate and fumarate while being able to generate ATP through electron transport phosphorylation coupled to fumarate reduction (Miwa Iwamoto et al., 2002). Nitrate and nitrite reduction may be stimulated by increasing the numbers of W. *succinogenes* present in the rumen (Miwa Iwamoto et al., 2002). The addition of 10 mM NO_3 to mixed ruminal microbes inhibited the growth of total bacteria in 12 and 24 h incubation and reduced total VFA production. It was reported that 4.8 mM of nitrite accumulation was observed after the addition of 10 mM NO_3 and this could have inhibited microbial growth. *Selenomonas ruminantium* was not affected by addition of nitrate or nitrite toxicity. *Veillonella parvula* and *Wolinella succinogenes* bacterial cell numbers increased after 12h (3.41 and 3.11 to 3.66 and 3.51 (log/ml), respectively), but decreased after 24h (3.66 and 3.51 to 3.18 and <2 (log/ml), respectively) probably due to the low availability of nitrate. This may mean that V. *parvula* and W. *succinogenes* depend on NO_3 reduction as an energy source (Miwa Iwamoto et al., 2002). These findings suggest that W. *succinogenes* and V. *parvula* may tolerate high NO_3 diets. The study indicated that CH_4 production by methanogens was inhibited by nitrite. This further supports the notion that methanogens are sensitive to nitrite. The direct inhibition of methanogenesis by nitrite is possible because methanogens acquire energy through electron transport phosphorylation. So the use of nitrate in combination with nitrate reducing microbes that utilize H_2 may lower the accumulation of H_2 and reduce ruminal methanogenesis (Miwa Iwamoto et al., 2002).

Dietary NO_3 has also been investigated *in vivo* in lactating Holstein-Friesian dairy cows fed corn silage (van Zijderveld, Gerrits, et al., 2011). Although the use of NO_3 requires an adaptation period to reduce the risk of methemoglobinemia and also been shown to be successful effective at reducing methanogenesis (van Zijderveld et al., 2011). The study used a nitrate source in the control (urea) and treatment (Calcinit) diet. The study showed that CH_4 production was reduced by as much as 16% when animals were fed a nitrate-based diet. Dry matter intake, milk yield, and gross energy intake were not affected by treatment. The protein content of milk was lowered when NO_3 was added to the diet. It was also noted that H_2 production was increased with the addition of NO_3 when compared to the control diet. Nitrate reduced CH_4 production, but was lower than the theoretical potential indicating that NO_3 was not completely reduced to ammonia (van Zijderveld, Gerrits, et al., 2011).

The ability of NO_3 to reduce methanogenesis in the animal may depend on propionogenesis since both of these processes require H_2 (van Zijderveld, Gerrits et al., 2011). However, there has been variation in the efficiency of NO_3 from sheep to dairy cows and this could be due to feed intake and retention- time and the pH of the rumen (van Zijderveld, Gerrits, et al., 2011). For example if NO_3 competes for reducing equivalents from propionogenesis then in sheep where propionogenesis plays a small role nitrate would be less effective than in a dairy cow where propionogenesis is a major pathway (van Zijderveld, Gerrits et al., 2011). This study suggests that NO_3 could be effective in animals fed at or near maintenance level. Nitrate was effective at reducing CH_4 emissions in dairy cows fed corn-silage based diets without affecting their digestibility or milk production (van Zijderveld, Gerrits, et al., 2011).

Hulshof et al. (2012) also evaluated the *in vivo* effect of dietary NO_3 on CH_4, VFA, and NH_3 concentrations in beef cattle fed sugar cane based diet. The effect of NO_3 in beef cattle has

not been extensively researched so this study provides insight to the impact dietary NO_3 has on beef cattle. The control diet contained urea while the experimental diet contained 22g NO_3/ kg DM of Calcinit. There was a decrease in DMI intake when NO_3 was added to the diet and rumen VFA concentrations were not affected by nitrate, which suggest that nitrite had little impact on microorganisms (Hulshof et al., 2012). The effect on NO_3 reducing fermentation gases can be easily counteracted with diets high in proteins. It was suggested that NO_3 should only be applied to diets that require a source of non-protein nitrogen (Hulshof et al., 2012). Total VFA concentrations were not affected by addition of NO_3. Compared to control sample the addition of Calcinit reduced propionate, while acetate increased. The acetate to propionate ratio was greater for NO_3 than the control and NH_3-N was greater for the animals receiving NO_3 indicating that NO_3 competes for H_2 with methanogenesis and propionogenesis (Hulshof et al., 2012). The study indicated that the animals on the NO_3 diet had a lower N efficiency and also to account for a variation in start times of consuming the diets offered to the control and nitrate treatment groups, which could explain the difference in the NH_3-N concentrations since it was iso-nitrogenous (Hulshof et al., 2012).

1.3.3 Effect of Feed Additives on Methane Production. The use of multiple feed additives may have an additive effect or may follow two different mechanisms in reducing methanogenesis can be of interest due to the concern of microbial adaptation to feed additives over time. The combination of nitrate with ß1-4 galacto-oligosaccharides (GOS) and nisin has been evaluated for their effect on methanogenesis (Sar et al., 2004). Evidence indicated that a diet rich in NO_3 can cause nitrite accumulation and methemoglobinemia in ruminants (van Zijderveld et al., 2011) and it has been shown that ß1-4 galacto-oligosaccharides can reduce methanogenesis and methemoglobinemia in sheep (Sar et al., 2004). Nitrate in combination with

nisin and GOS decreased plasma nitrite concentrations. Compared to NO_3 alone, combination of NO_3 with nisin and GOS lowered rumen NO_3 concentrations and increased the rate of NO_3 disappearance in the rumen. The mean rumen pH was also lower for NO_3 than the control. The rumen NH_3-N increased after the addition of NO_3 alone and in combination with GOS and nisin. Nitrate alone has been shown to reduce methanogenesis; however the anxiety of methemoglobinemia in ruminants is of great concern. Therefore the addition of NO_3 in combination with other feed additives that may increase the clearance of nitrate from the rumen is of great interest.

The effect of NO_3 and sulfate were also evaluated *in vivo* in lambs fed a corn-silage based diet (van Zijderveld et al., 2010). Theoretically one mole of CH_4 can be reduced by one mole of NO_3 and reduced further to NH_3 (van Zijderveld et al., 2010). The production of ammonia is beneficial to animals because it can serve as source of fermentable protein for low protein diets.. The results of the study revealed that dietary NO_3 and sulfate reduced CH_4 production. Also, while addition of nitrate increased total number of methanogens, sulfate addition increased total number of methanogens (van Zijderveld et al., 2010). The decrease could be explained by a decrease in H_2 availability. The protozoal population in the study was not affected by the addition of NO_3 or sulfate. However, CH_4 production was lower decreased with nitrate and the combination of nitrate-sulfate compared to control (van Zijderveld et al., 2010). Sulfate may play a role in improving the rate of reduction of nitrite since methemoglobinemia was not detected in control or samples that contain sulfate (van Zijderveld et al., 2010). Sulfate can also be effective as NO_3 in reducing methanogenesis by using H_2 when it is converted to hydrogen sulfide, but research has shown that it needs a longer period of time than NO_3 to show this effect (van Zijderveld et al., 2010).

There has been interest in combining feed additives to effectively reduce methanogenesis without disrupting normal rumen fermentation of feedstuffs. Iwamoto et al. (1999) evaluated the effects of NO_3 combined with fumarate on *in vitro* CH_4 production, fermentation, and cellulose digestion by mixed-rumen microorganisms and it was observed that addition of fumarate to nitrate did not reduce VFA production or digestion. It has been reported that fumarate can reduce *in vitro* CH_4 and increase propionate productions (Asanuma et al., 1999). The accumulation of nitrite is a concern due to the fact that the rate of NO_3 reduction is greater than reduction of nitrite (M. Iwamoto, Asanuma, & Hino, 1999). Although van Zijderveld, (2010 and 2011) has noted that an adaptation to NO_3 in the diet by ruminants is sufficient to avoid toxic effects, the enhancement in the reduction of nitrite is needed in diets that contain NO_3 (M. Iwamoto et al., 1999). In this particular study the rate of NO_3 reduction decreased over time resulting in the accumulation of nitrite, which may have inhibited nitrate-reducing bacteria. Fermentation and total VFAs were suppressed by the addition of NO_3. The addition of fumarate enhanced the reduction of nitrate. This suggests that fumarate does not compete with nitrate for electrons. Possibly the activity and growth of NO_3 and fumarate reducing bacteria is enhanced by fumarate. Fumarate reduction has been shown to be associated with regeneration of ATP from electron transport phosphorylation (Asanuma et al., 1999). The addition of fumarate to NO_3 did not affect VFA production or digestion. However when combined these two feed additives may decrease methanogenesis without affecting a rumen fermentation (M. Iwamoto et al., 1999).

1.4 Summary

Enteric CH_4 production contributes significantly to agricultural based CH_4 emissions, which may contribute to the overall global GHG emissions. It is well known that nutritional

strategies have been investigated as a method to reduce CH_4 production from ruminant animals. Although many feed additives like fats/essential oils (Spanghero et al., 2008), plant secondary metabolites (Patra & Saxena, 2010), and defaunation by ionophores (Grainger et al., 2010) have all reduced enteric CH_4, however there has been a decrease in acetate production and DMI. Also the use of these feed additives has led to microbial adaptation and most importantly their effect on enteric CH_4 has varied. The use of nitrate in the ruminant diet has given promise to reduce enteric CH_4 production by acting as an electron acceptor (Hulshof et al., 2012). However it comes with the increased risk of nitrate/nitrite accumulation in the rumen. The use of fumarate as a free acid and encapsulated (Wood et al., 2009) has shown to reduce CH_4 production, while authors like (Beauchemin & McGinn, 2006a; Beauchemin & McGinn, 2006b) reported had no effect on CH_4 production. However authors have combined nitrate with other feed additives (Iwamoto et al., 1999; Sar et al., 2004; van Zijderveld et al., 2010) to reduce CH_4 and minimize the adverse effects nitrate may have on the ruminant.

In addition to feed additives the manipulation of the feed before it is fed to the animal may also have an impact on methanogenesis. Forage processing can impact the rate of passage of feed, which may affect CH_4 production (Johnson & Johnson, 1995).The particle size of feed can impact VFA production and the pH (Teimouri Yansari et al., 2004), which impacts the rumen environment and microorganisms that are involved in methanogenesis. Also the knowledge that has been gained about the characteristics of methanogens and the substrates they utilize to reduce CO_2 in the rumen has led to studies that have focused on how feed additives affect their functions in the rumen (Zhou et al., 2011). It is important to investigate how the manipulation of feed by altering the particle size and the use of nitrate and fumarate can affect *in vitro*

fermentation and CH_4 production to understand how these strategies may impact methanogenesis *in vivo*.

Conclusion

Methane formation is an inevitable process that occurs during enteric fermentation and cannot be completely eliminated without possibly causing an adverse effect on fermentation and ultimately the ruminant animal. Therefore, the use of feed additives with antimethanogenic or electron accepting properties in the diet of ruminant animals is a growing area of interest to reduce enteric CH_4 production.

CHAPTER 2

Effects of Feed Particle Size and Fumarate or Nitrate on Methanogens and *in vitro* Methane Production

Abstract

This study investigated the effects of feed particle size and nitrate or fumarate alone or in combination on *in vitro* CH_4 production and the abundances of methanogens. Rumen fluid was collected from a Holstein-Friesian dry cow and steer (experiment 1) and a dry cow and two steers (experiment 2) averaging 625.4 kg in BW. Animals were offered 11.4 kg/d per animal of concentrate diet containing equal amounts of soybean meal, whole cottonseed, and ground corn once a day with grass hay *ad libitum* (experiment 1) and 15.9 kg/d of TMR (experiment 2). Feed grab samples were collected, oven-dried, coarsely pulverized in a regular kitchen blender for one minute, and separated into three particle-sizes (PS; 0.85, 1.4 and 2.36 mm). The feed additives used were nitrate, fumarate and a nitrate-fumarate combination. Total microbial DNA was extracted from *in vitro* rumen fluid samples and PCR was performed to amplify genomic DNA. Real-time PCR was used to quantify the relative abundances of methanogens. The results obtained shows that nitrate reduced ($p < 0.05$) CH_4 production by 57% (experiment 1) and 59% (experiment 2), while the nitrate-fumarate combination reduced ($p < 0.05$) CH_4 production by 40% (experiment 1) and 68 % (experiment 2). Fumarate had no effect on CH_4 production. In experiment 1, CH_4 production for the medium PS was 31% and 39% lower ($p<0.05$) than the small and large PS. Methane production was 17% and 16% higher ($p<0.05$) for the large PS compared to the small and medium PS (experiment 2). The addition of all feed additives had no effect on total methanogens. Fumarate decreased ($p<0.05$) the abundance of *Methanobrevibacter sp.* AbM4 and nitrate decreased ($p<0.05$) the abundance of M. *stadtmanae*. Based on the results

observed nitrate and the nitrate-fumarate combination may be used to reduce methane production. The effects of nitrate and fumarate on methanogens were species specific and if used in combination could be more effective in targeting a broader range of methanogen species.

Keywords: *in vitro*, *methane, nitrate, fumarate, particle size, methanogens*

2.1 Introduction

Enteric CH_4 formation from ruminants is a concern in agricultural and environmental research due to increased awareness of climate change. Climate change can have direct and indirect consequences to the economy, but most importantly significant economic costs in agriculture (Moss, Jouany, & Newbold, 2000). Ruminant livestock account for 20% of the U.S. CH_4 emissions (EPA, 2012). Methane that is exhaled from the ruminant is a loss of feed-derived energy to the animal.

Diet plays a major role in the variability of CH_4 loss to the animal and there are two major mechanisms: the rate and passage of fermentable carbohydrate and hydrogen supply in relation to VFA production (Johnson & Johnson, 1995). Methane production in the rumen can be altered by different strategies like the use of rumen modifiers. The level of feed intake, feed type, and feed additives all impact the amount of CH_4 produced by ruminants (Johnson & Johnson, 1995). Therefore, introducing feed additives that generate H_2 utilizing reactions or alter the rumen to reduce concentrations of H_2 in the rumen into the ruminant diet is one of several means researchers have proposed to decrease the ability of methanogens to produce CH_4. For example, fumarate, a dicarboxylic acid is a key intermediate in rumen microbial metabolism (Yu et al., 2010) and has long been proposed as a rumen modifier by enhancing the succinate-propionate pathway (Araújo et al., 2011). Several investigators (Asanuma et al., 1999; Bayaru et al., 2000; Mao et al., 2008; Abdl-Rahman et al., 2010; Wood et al., 2009; Yu et al., 2010) have

also used fumarate as a feed additive to alter rumen fermentation and as an electron acceptor to reduce methanogenesis and increase VFA production by increasing propionate. Nitrate has also been used as a feed additive to reduce enteric CH_4 production *in vitro* and *in vivo* (Bozic et al., 2009 and Hulshof et al., 2012). However the concern for the accumulation of nitrite in the rumen when nitrate is used as a feed additive has led to the use of nitrate in combination with other feed additives like sulfate (van Zijderveld et al. 2010) and other feed additives that may increase the rate of nitrate and nitrite reduction (Iwamoto et al., 1999). In addition to rumen modifiers the particle size of the feed can affect enteric CH_4 production. Johnson & Johnson (1995) indicated that nearly 28% of variation in CH_4 production in the literature was related to the passage rate of the feed.

Methanogens belong to the domain Archaea and are established in the rumen shortly after birth (Hook et al., 2010). Methanogens are responsible for the production of CH_4, a terminal step in fermentation, in ruminant animals by a process called methanogenesis (Hook et al., 2010). The substrates methanogens use for this process can vary between the methanogen species (Leahy et al., 2010). Therefore, understanding their response to changes in the diet of a ruminant animal is essential to finding new strategies to reduce enteric CH_4 formation. *Methanobrevibacter* is the dominant group of methanogens in the bovine rumen of dairy cows followed by *Methanosphaera stadtmanae* (Whitford et al., 2001; Skillman et al., 2006; Hook et al., 2010). *Methanosphaera stadtmanae* and *Methanobrevibacter sp*. AbM4 are both hydrogenotrophic and can use H_2 as s substrate for methanogenesis (Leahy et al., 2013). However M. *stadtmanae* can only use methanol as an alternative substrate for methanogenesis and acetate as an energy source (Fricke et al., 2006) while *Methanobrevibacter sp*. AbM4 can also use CO_2 and formate (Leahy et al., 2013) to synthesize CH_4. *Methanobrevibacter sp*. AbM4 has a similar methanogenesis

pathway to *Methanobrevibacter ruminantium* M1 indicating that these two species can use the same substrates to synthesize CH_4 (Leahy et al., 2013).

Methanogen numbers have been known to decrease by the addition of methanogen inhibitors that can alter the rumen environment (Knight et al., 2011). The knowledge of substrate preference for methanogens to produce CH_4 can be species specific (Hook et al., 2010) and is crucial to increase the efficacy of methane inhibitors. The addition of disodium fumarate has been reported to reduce methanogen populations (Zhou et al., 2012) and this effect can depend on the association of the methanogen with fluid or solid fractions in rumen fluid suggesting that methane inhibitors not only influence the methanogen species but their efficacy can be an indirect effect by influencing microbial shifts in other populations in the rumen (Asanuma et al., 1999). The addition of nitrate has also been shown to inhibit CH_4 production and can reduce total methanogens by 97% (Zhou et al., 2011). The effects of nitrate and its intermediates (nitrite and nitrous oxide) on methanogens commonly found in rice field soils (Methanosarcina barkeri and Methanobacterium bryantii) varied among the species (Kluber & Conrad, 1998) further suggesting that methane inhibitors target methanogens on the species level.

In the current study we investigated the potential benefit of combining nitrate with fumarate to reduce *in vitro* CH_4 production and evaluated if the mixture could be as effective as or better than nitrate or fumarate alone. Also we determined if the particle size of the feed had any influence on CH_4 production. Lastly, we examined if the feed additives had any effect on the abundance of methanogens. Therefore, it is hypothesized that nitrate and fumarate reduces in vitro CH_4 production and that the mixture of nitrate-fumarate will provide a greater reduction *in*

vitro CH_4 production by reducing the availability of H_2 for methanogenesis while decreasing the abundance of methanogens.

2.2 Materials and Methods

2.2.1 Experimental Overview. The experiment was approved by the North Carolina Agriculture & Technical State University Institutional Review Board (13-0275). Two experiments were conducted to investigate the effect of nitrate, fumarate, and nitrate-fumarate combination with three feed particle sizes (PS) as large, medium, and small (2.36, 1.4, and 0.850mm) on the abundance of methanogens and *in vitro* CH_4 production. The experiments are summarized in table 3. Experiment 1 consisted of two Holstein-Friesian cattle (steer and non-lactating cow) averaging 650 kg. The diet consisted of a daily feed allowance of 11.4 kg/d per animal of equal amounts of soybean meal, whole cottonseed, and ground corn once a day. The animals were offered free access to grass hay and when grass was not available in the winter months and they were allowed to graze on forage in the spring. The particle sizes (PS) used for each experiment were 2.36 mm, 1.4 mm, and 0.850 mm. Experiment 2 the animals consisted of the same cow and two new steers. The diet consisted of a totally mixed ration (TMR) of silage, hay, corn, corn gluten meal, soybean meal, and mineral supplements totaling 15.9 kg/d per animal. All diets were formulated to meet the nutrient requirements of all animals.

2.2.2 Animals Used in the Study. Animals were provided and housed at the North Carolina Department of Agriculture and Consumer Services (NCDA&CS) Piedmont Research Station in Salisbury, NC. The experimental animals in the two studies consisted of 4 Holstein-Friesian cattle (dry cow and three steers) average body weight 625.4 kg. The animals were used as rumen fluid donors for all *in vitro* work. In the first experiments, a cow and a steer served as

rumen fluid donors. For the second experiment the same cow from experiment 1 and two new steers were used as rumen fluid donors (Table 3).

Table 3

Summary of Experiments

	Experiment 1	**Experiment 2**
Date	January 2012-August 2012	January 2013-August 2013
Number of animals	dry cow and steer avg. BW:650 kg	dry cow; steer, n=2; avg. BW: 625.4
Diet	11.4 kg/d soybean meal, whole cottonseed, ground corn, and grass hay ad libitum in winter and grazing in summer	TMR: 15.9 kg/d silage, hay, corn, corn gluten, soybean meal, and minerals
Feed additives, g **Particle size, mm**	Nitrate, Fumarate, Nitrate-Fumarate Small, Medium, Large	Nitrate, Fumarate, Nitrate-Fumarate Small , Medium, Large
Measurements	CH_4, chemical composition of feed , relative abundance of methanogens	CH_4, chemical composition of feed , relative abundance of methanogens

Note: Small, medium. Large PS (0.85, 1.4, 2.36 mm, respectively)

2.2.3 Sampling. Rumen contents were obtained from the rumen of 4 ruminally cannulated (Bar Diamond, Idaho, US) dairy cattle. Samples were collected every Tuesday at 0900h after 17 h of fasting. Prior to collecting rumen contents each thermos flask was filled with warm water to ensure the temperature inside the thermos was suitable enough for microorganisms being transferred from the rumen to the thermos. The animals were placed in the head gate for sample collection. Rumen digesta from previous feedings were partially removed to thoroughly mix the rumen contents. The mixed rumen contents were removed using a 250 mL plastic beaker, strained through four layers of cheesecloth and drained to the top of the

empty thermos to minimize oxygen in the headspace, primed with CO_2 and sealed tightly prior to being transported to the university laboratory for analysis.

2.2.4 Feed Analysis. Table 4 shows the chemical analysis of the different feed particle sizes used in the study. Chemical composition was determined after samples were oven dried 70°C for 24 h. Dry matter was determined in duplicate by weighing 2.0 g of feed into pre-dried crucibles and dried in the oven at 100°C overnight to constant weight, removed and allowed to cool in a desiccator. Sample weights were determined the next day on the Ohaus Explorer Pro balance (Parsippany, NJ, USA). The ash content was determined by placing the feed samples from the dry matter experiment in crucibles into the furnace until internal temperature reached 550°C for one hour and then weighed for ash content. Neutral detergent fiber (NDF) using α-amylase and sodium sulfite and acid detergent fiber analysis(ADF) was also determined by grinding the feed samples to 1mm and 0.50 g of the air dry feed was placed into F57 fiber filter bags (ANKOM Technology, Macedon, NY) and heat sealed. The extraction method for determining neutral and acid detergent fiber provided by ANKOM Technology was followed to complete the process. After the procedure the sample bags were placed in the oven at 70°C and were dried overnight. The next morning the sample weight was determined using the Ohaus Explorer Pro balance (Parsippany, NJ, USA). Crude Protein of feed samples was determined on the TruSpec CN (Leco Corporation, Michigan, US). Briefly feed samples (0.1g) were placed in foil cups purchased from the manufacturer (Leco Corporation, Michigan, US) and analyzed for protein in triplicate with EDTA as the standard and carrier gases were compressed air, Helium, and Oxygen. The procedure for sample preparation and analysis was followed according to the recommendation from the manufacturer.

Table 4

Chemical Composition of Experimental Diet by Particle Size

	Experiment 1			Experiment 2		
Particle size , mm	Large	Medium	Small	Large	Medium	Small
Dry matter, %	94.71	89.52	90.90	89.62	91.08	89.92
Total Ash, %	12.5	3.13	12.4	3.13	3.13	3.10
Crude protein[a], %	12.38	-	-	11.98	-	-
aNDF %	56.28	46.28	26.73	54.86	44.59	26.23
ADF %	29.68	25.16	13.77	33.95	26.86	14.15
Ca, ppm	23.4	33.2	37.8	17.9	19.6	38.4
K, ppm	46.3	45.6	33.6	50.4	39.3	42.0
Mg, ppm	10.0	10.1	8.6	12.2	9.7	9.6
P, ppm	23.0	28.2	18.8	22.4	19.8	20.9

[a] Feed sample before separated into particle size; aNDF (neutral detergent fiber); ADF (acid detergent fiber); Ca (calcium), K (Potassium), Mg (Magnesium), P (Phosphorus)

2.2.5 In vitro Method for the Determination of Methane Production. Prior to start of the experiment feed samples for the experiment were collected from the feed trough and oven dried at 70°C for 24 h. Feed grab samples was coarsely pulverized in a kitchen blender for one minute and separated into three (PS) 2.36, 1.4, and 0.850 mm using standard test sieves (Fisher Scientific Company, US). The in vitro method for the determination of CH_4 production was carried out according to the first stage *in vitro* digestibility method of Tilley and Terry, 1963. The artificial saliva was prepared according to McDougall, 1948 and stored overnight at 39 °C:

Strained rumen fluid and artificial saliva was mixed in 1:4 ratios in 500 ml Erlenmeyer flasks (240 ml of buffer and 60 ml of ruminal fluid for a total volume of 300 ml in triplicates). A 5g feed sample and 1g of calcium nitrate and calcium fumarate (Fisher Scientific, US) was added to each flask in triplicates (Table 5). After the addition of all reagents to each flask; the flasks

were primed with CO_2 once more to maintain anaerobic conditions and incubated at 39°C for 48 hours.

Table 5

Summary of the Feed to Feed Additive Ratio Used for in vitro CH_4 Production

Sample IDs (in triplicates)	Feed Additives	Feed Additives (grams)	Feed Sample (grams)
1-3	Control	n/a	5g
4-6	$CaNO_3$	1g	5g
7-9	$CaC_4H_2O_4$	1g	5g
10-12	*N+F Mixture	1g (50/50)	5g
13-15	Blank	n/a	n/a

2.2.6 Determination of in vitro Methane Production. Following the end of the 48 h incubation flasks were removed from the oven. The samples were analyzed for CH_4 concentrations on the G2301 Picarro CH_4 and CO_2 Gas Analyzer (PICARRO®; CA, USA). *In vitro* flasks were removed from the oven and placed in the refrigerator for 10 minutes to stop fermentation. The headspace from each flask was directly connected into the analyzer and the numerical value and peak on the chromatogram displayed on the monitor of the computer indicated the amount of CH_4 detected (Picarro G2301 Analyzer User's Guide, 2011).

2.2.7 Microbial DNA Extraction. Rumen contents from four Holstein-Friesian dairy animals (non-lactating cow and 3 steers) were removed via rumen cannula (Bar Diamond, USA) and used for the *in vitro* fermentation technique (Tilley and Terry, 1963; McDougall, 1948). Rumen fluid samples (30mL) were collected from the *in vitro* fermentation flasks 48 hours after incubation by directly transferring the fluid mixture into 50 mL conical vials and stored at -20°C until further analysis. DNA was extracted from rumen fluid samples using the QIAamp® DNA Stool Mini Kit (Qiagen, USA), which is a silica membrane based technique that purifies

genomic, bacterial, viral DNA in a volume of 200 µl. The kit was used as recommended by the manufacturer with a modification in the temperature used to lyse the bacterial cells. Briefly, rumen contents were mixed with a hand held homogenizer to ensure the samples were thoroughly mixed to include free-living methanogens and methanogens associated with the solid fraction layers. An aliquot of 200µl of the homogenized rumen contents was collected from each sample and combined with the buffer ASL and heated to 95°C for five minutes to lyse gram-positive and gram-negative bacteria (70°C). The samples were centrifuged at full speed of 16,000 x g for one minute to remove particles. Potential inhibitors were removed using InhibitEx (an adsorption resin) tablets and samples were treated with proteinase K and buffer AL and incubated at 70°C for ten minutes to remove any possible degrading proteins. Ethanol was used to precipitate the DNA and was applied to the QIAamp spin columns. The columns were washed with 500 µl of buffers AW1 and AW2 per instructions by the kit manual. The spin column was then washed with 200µl of the buffer AE incubated at room temperature for one minute and centrifuged at full speed for one minute to elute DNA. The DNA quality and concentrations were determined at absorbencies of 260 and 280 nm on the Nanodrop spectrophotometer ND-1000 (NanoDrop Technologies, US). Total DNA concentrations were calculated from the DNA concentrations recorded on the Nanodrop and the final concentration volume yielded by the DNA extraction kit.

2.2.8 Amplification of Methanogenic DNA using PCR. Genomic DNA isolated from rumen fluid samples using the QIAamp DNA Stool Mini kit was polled and used for PCR amplification to detect the presence of total methanogens, *Methanobrevibacter* sp. AbM4 and *Methanosphaera* stadtmanae using glyceraldehydes-3-phosphate (GAPDH) as the control to validate the PCR experiment. Primers to target the methanogen 16S rRNA gene for the study

were selected from previous studies conducted by Zhou et al.,(2009) and Mohammed et al., (2011) who designed the primers based on the alignment of the identified targeted species sequences and the 16S rRNA gene sequences available in Genbank. All of the primers used in the current study were synthesized by MWG Biotech (MWG Biotech, Inc., NC). A Qiagen Multiplex PCR kit (Qiagen, USA) was used for PCR preparation using one primer per sample without utilizing the multiplex feature for PCR amplification. According to the manufacturer, 25 µl of the master mix along with 2.5 µl of the forward primer and 2.5 µl of the reverse primer was added to each tube. Each sample contained 250 ng of template DNA in each PCR reaction tube (Table 7). Also RNase-free water provided by the manufacturer was added to each reaction tube to give a total volume of 50 µl. The reaction tubes were mixed briefly using a vortex and placed in a thermocycler (MWG-Biotech Inc., Primus 96, USA) and processed according to a program that was created for the specific primer pairs used in the study (Zhou et al., 2009 and Mohammed et al., 2011). Conventional PCR was performed with the following program: at 95°C for 30s, 30 cycles at 60°C for 90s, extension at 68°C for 60s, and 1 cycle of final extension at 60°C for 7 min to amplify the template DNA. After amplification, the amplicons were ran on a 1% agarose gel (0.5g in 50 ml x TBE) using DNA markers for electrophoresis and stained with ethidium bromide. Table 6 illustrates the components and amounts per the manufacturer's requirements in each reaction tube. The primer sequences and expected length (bp) of PCR products used in the study are described in table 7.

Table 6

Qiagen Multiplex PCR Components

Component	Volume/reaction	Final concentration
2X QIAGEN Multiplex PCR Master Mix	25µl	1x
10x Primer mix	5µl	0.2µM
RNase-free water	Variable	-
Template DNA	Variable	250 ng/ µl
Total Volume	50µl	50µl

Table 7

Primers Used in the Study for Conventional PCR and qRT-PCR Analysis

Methanogen Species	Primer	Sequence (5' to 3')	Expected size(bp)	Reference
Methanobrevibacter sp. Strain AbM4	AbM4-F AbM4-R	TTTAATAAGTCTCTGGTGAAATC AGATTCGTTCTAGTTAGACGC	160	Zhou et al. 2009
M. stadtmanae	Stadt-F Stadt-R	CTTAACTATAAGAATTGCTGGAG TTCGTTACTCACCGTCAAGATC	150	Zhou et al. 2009
Total Methanogens	Met 1-F Met 1-R	CGATGCGGACTTGGTGTTG GTTTCAGTCTTGCGACCGTACTT	98	Mohammed et al. 2011
GAPDH	Forward Reverse	GGTCGGAGTCAACGGATTTGGTCG CCTCCGACGCCTGCTTCACCAC	240	Wang et al. 2008

Note: F- designates the forward primer and R -designates the reverse primer

2.2.9 Real-Time Polymerase Chain Reaction to Determine the Relative Quantification of *in vitro* Methanogen Populations.

DNA isolated from rumen fluid samples using the QIAamp DNA Stool Mini kit was used for amplification to determine the relative quantification of methanogens using the internal control gene GAPDH and the untreated control as the reference gene. Glyceraldehyde-3-phosphate dehydrogenase was used as a stable housekeeping gene since it is found in Archaea and catalyzes gluconeogenic reactions (Costa, Lie, Jacobs, & Leigh, 2013). The SsoAdvanced™ SYBR® Green Supermix (Bio-Rad

Laboratories, Hercules, Ca.) were used for real-time PCR. According to the manufacturer 1.5µl of the forward and reverse primers (Table 8), 50 ng of gDNA per sample was added to a 96-well tray along with 10 µl of SsoAdvanced™ SYBR® Green Supermix in duplicates. Lastly RNase-free water was added to each well for a total of 20 µl per well. The tray was centrifuged briefly to ensure all contents in the wells were thoroughly mixed. Following centrifugation, the PCR tray was placed in the CFX Connect thermocycler (Bio-Rad, Hercules, CA) and the PCR cycling conditions were set using the manufacturer's supplied software. The threshold cycle for each well was determined using the provided real-time cycler software. Real-Time PCR was performed with the following program: 95°C for 10 min., followed by 40 cycles at 95°C for 3 s and 60°C for 30s, and melting curve detection at 95 for 5 s (Zhou et al., 2009 and Mohammed et al., 2011).

The relative gene abundance of the 16S rRNA genes for total methanogens, *Methanobrevibacter* sp. AbM4 and *Methanosphaera* stadtmanae based on real-time PCR data was quantified and analyzed using the comparative C_T method ($2^{-\Delta\Delta C}{}_T$ method; Livak & Schmittgen, 2001). The untreated control used in the study was the control *in vitro* fermentation sample and the reference control used in the study was GAPDH. The target genes in the study were nitrate, fumarate, and nitrate-fumarate treated *in vitro* samples. Since real-time PCR samples were ran in duplicates the mean was calculated for each quantitation cycle (Cq) before performing any further calculations. The mean C_T of the reference genes (sample control) and target genes (nitrate, fumarate and nitrate/fumarate) were normalized to that of the reference gene (GAPDH). Then the ΔC_T of the test sample and the calibrator sample was normalized and then the normalized expression ratio was calculated as $2^{-\Delta\Delta C}{}_T$. The calculation steps were as follows (Livak & Schmittgen, 2001):

$$\Delta C_T \text{ (test)} = \text{(target control} - \text{GAPDH)} \quad \text{(Eq. 5)}$$

$$\Delta C_T \text{ (calibrator)} = \text{(target gene} - \text{GAPDH)} \quad \text{(Eq. 6)}$$

$$\Delta\Delta C_T = \Delta C_T \text{ (test)} - \Delta C_T \text{ (calibrator)} \quad \text{(Eq. 7)}$$

$$2^{-\Delta\Delta C_T} = \text{fold change due to treatment} \quad \text{(Eq. 8)}$$

2.3 Experimental Method and Statistical Analysis.

2.3.1 Experiment. The experimental for the study involved three PS (2.36, 1.4, and 0.850 mm) of the experimental diets and three feed additives (nitrate, fumarate, nitrate-fumarate) as treatments and a control.

2.3.2 Statistical Analysis. Data for the *in vitro* CH_4 production and chemical composition of the feed were analyzed using SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). The data for CH_4 production were combined for each animal. The experiment was conducted in a randomized complete block design with animals as blocks, and three replications of the PS and feed additive treatments per block. Methane production data were analyzed with mixed procedure (PROC MIXED) of SAS, using a mixed model with animals as a random effect and particle size and feed additive treatments as fixed effects (Little, Milliken, Stroup, Wolfinger, & Schabenberger, 2006; SAS Institute, 2012). The particle size by feed additive interaction and the fumarate by nitrate interaction were examined and where this interaction was not significant the sample treatments were compared independently of feed PS. The Kruskal-Wallis one way analysis of variance test was performed using GraphPad PRISM® to analyze if DNA concentrations. All treatment effects were significant at $p < 0.05$ and probability values between 0.06 and 0.10 were considered trends.

2.4 Results

2.4.1 Effect of Nitrate, Fumarate, and Nitrate-Fumarate on CH_4 Production. Table 8 shows the effect of feed additives on CH_4 production in experiment 1 and 2. In experiment 1, the addition of nitrate decreased CH_4 production by 57% ($p<0.05$) compared to the control and fumarate, however the addition of fumarate had no effect on CH_4 production compared to the control and the nitrate-fumarate combination. The addition of the nitrate-fumarate combination also decreased ($p<0.05$; 40%) CH_4 production by 40% compared to the control or fumarate, but there was no significant difference between nitrate and the nitrate-fumarate combination. In experiment 2, addition of nitrate decreased ($p<0.05$) CH_4 production by 59% compared to the control or fumarate, but there were no significant differences between nitrate and the nitrate-fumarate combination, and between fumarate and the control. The nitrate-fumarate combination also decreased CH_4 production by 68% compared to the control or fumarate. The addition of nitrate and nitrate-fumarate combination consistently reduced CH_4 production in both experiments. Although the decrease in CH_4 production for the nitrate-fumarate combination in experiment 2 was 53% higher than in experiment 1, the experiment to experiment variation in CH_4 reduction for nitrate was much smaller (16%) than in the nitrate-fumarate combination.

Table 8

Effect of Nitrate, Fumarate, and Nitrate-Fumarate on CH_4 Production

CH_4, ppm	Control	Nitrate	Fumarate	Nitrate-Fumarate	SEM
Experiment 1	32.5^a	14.1^b	31.3^a	19.4^b	4.51
Experiment 2	28.5^a	11.8^b	28.7^a	9.2^b	5.25

Note. Different superscripts with in a row indicates means that are different ($p<0.05$)

2.4.2 Effect of Particle Size on CH_4 Production. Table 9 shows the effect of feed particle size on CH_4 production in both experiments. In experiment 1, CH_4 production for the

medium PS was lower ($p<0.05$) than the small and large PS, but with no significant difference between the small and large PS. In experiment 2, even though there was no significant difference between the small and the medium PS, CH_4 production for the large PS increased ($p<0.05$) by 17% and 16% compared to the medium and small PS, respectively. In other words, even though the effect of PS on CH_4 production was evident, there was no definite CH_4 production pattern that could be associated with any specific particle size used in the study.

Table 9

Effect of Feed Particle Size on CH_4 Production

PS, mm	S	M	L	SEM
CH_4, ppm (Exp.1)	25.8^a	17.7^b	29.2^a	3.4
CH_4, ppm (Exp. 2)	17.2^a	17.9^a	20.7^b	1.07

Note. Different superscripts with in a row indicates means that are different ($p < 0.05$)

2.4.3 Effect of Feed Additives on Total Microbial DNA Concentrations. Figure 3 shows the effect of feed additives on total microbial DNA in experiments 1 and 2. The concentrations of total microbial DNA among the feed additives were not different ($p = 0.9192$). Therefore, neither the individual feed additives nor their combination had any significant effect on total microbial DNA concentrations.

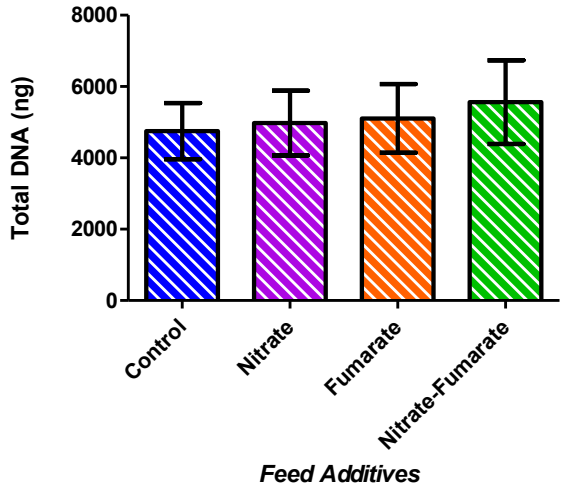

Figure 3. Effect of Feed Additives on Total Microbial DNA Concentrations

2.4.4 Detection of Methanogenic DNA. Figures 4-6 shows the 1% agarose gels for total and specific methanogens. Genomic DNA from methanogens in experiments 1 and 2 was extracted using the QIAamp DNA Stool Kit and amplified using the primers AbM4 F/r for *Methanobrevibacter sp. AbM4* and bands were visible for all samples at the expected molecular weight 160 (Zhou et al., 2009). Figure 4 shows the gel electrophoresis for *Methanobrevibacter sp. AbM4* with visible bands at the expected 160 base pair.

Genomic DNA was also amplified using the primers Stadt F/r for *Methanosphaera stadtmanae. AbM4* and bands were visible for all samples at the expected molecular weight 150 (Zhou et al., 2009). Figure 5 shows 1% agarose gel for *Methanosphaera stadtmanae* with visible bands at the expected molecular weight 150 (Zhou et al., 2009). Also extracted DNA was amplified using the primers Met1F/r for total methanogens yielded a band and was visible at the expected molecular weight 98 (Mohammed et al., 2011; Figure 6).

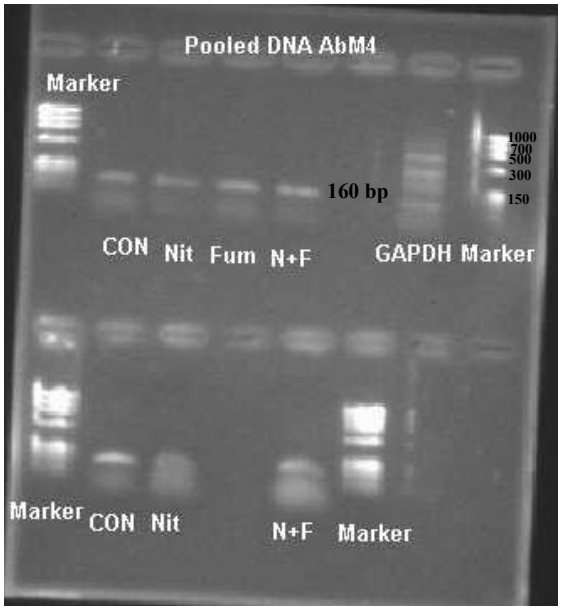

Figure 4. Detection of 16S rRNA gene for *Methanobrevibacter sp. AbM4*

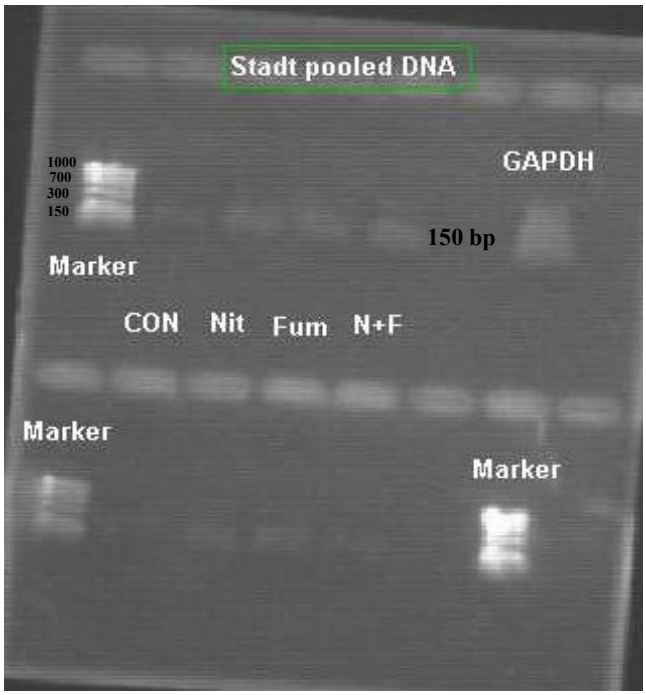

Figure 5. Detection of 16S rRNA gene for *Methanosphaera stadtmanae*.

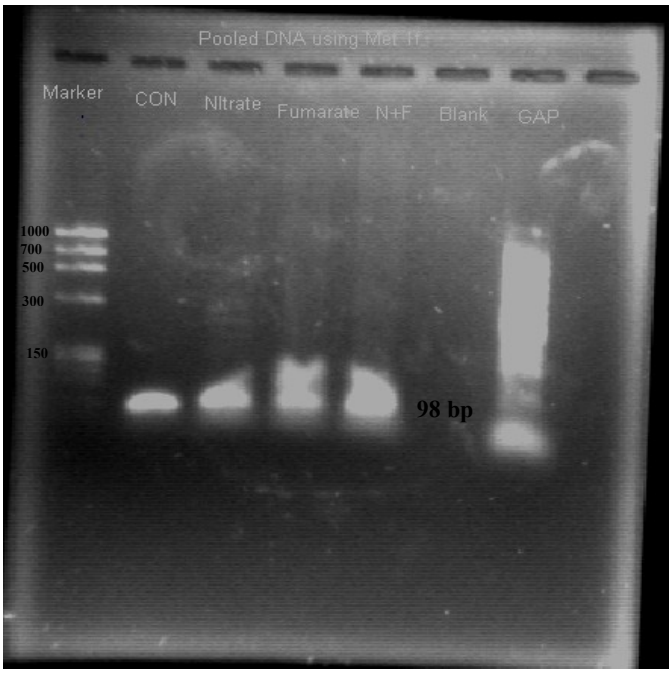

Figure 6. Detection of 16S rRNA gene for Total Methanogens.

Amplified products from real-time PCR for the primers AbM4F/r and Stadt-F/r were commercially sequenced (Eurofins MWG Operon, Huntsville AL). The similarities for all amplified methanogen DNA was determined using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare nucleotide sequences to sequences in the database to determine the closest methanogen. Microbial nucleotide (BLASTn) parameters were searched against complete genomes and optimized for highly similar sequences using megablast. The BLASTn results revealed PCR products sequenced with the primer for *Methanobrevibacter ruminantium sp. AbM4* were 98% and 90% with e-values of 6e-46 and 1e-33, respectively similar to Methanobrevibacter smithii and Methanobrevibacter ruminantium M1 strain with, respectively, while the sequences for Methanosphaera stadtmanae were 93% similar to Methanosphaera *stadtmanae DSM,* and 86% similar to an uncultured Methanosphaera sp. Clone with e-values from 2e-44 and 3e-07, respectively (see Appendix C). Therefore the primers used in the study were determined to be suitable to amplify methanogen DNA.

2.4.5 Evaluation of the Relative Abundance of Methanogenic DNA. Figures 7 and 8 show the effect of nitrate, fumarate and nitrate-fumarate combination on the relative abundance of *Methanobrevibacter sp. AbM4* and *Methanosphaera stadtmanae.* The fold change in DNA was used to determine the relative abundances of *Methanobrevibacter sp*. *AbM4* and *Methanosphaera stadtmanae*. Figure 7 shows that the abundance of *Methanobrevibacter sp*. AbM4 DNA increased ($p<0.05$) by the application of nitrate alone or in combination with fumarate compared to the addition of fumarate. However there was no significant difference in the fold change of DNA between nitrate and the nitrate-fumarate combination. Therefore, only the addition of fumarate had the greatest inhibition on the relative abundance of *Methanobrevibacter sp. AbM4*.

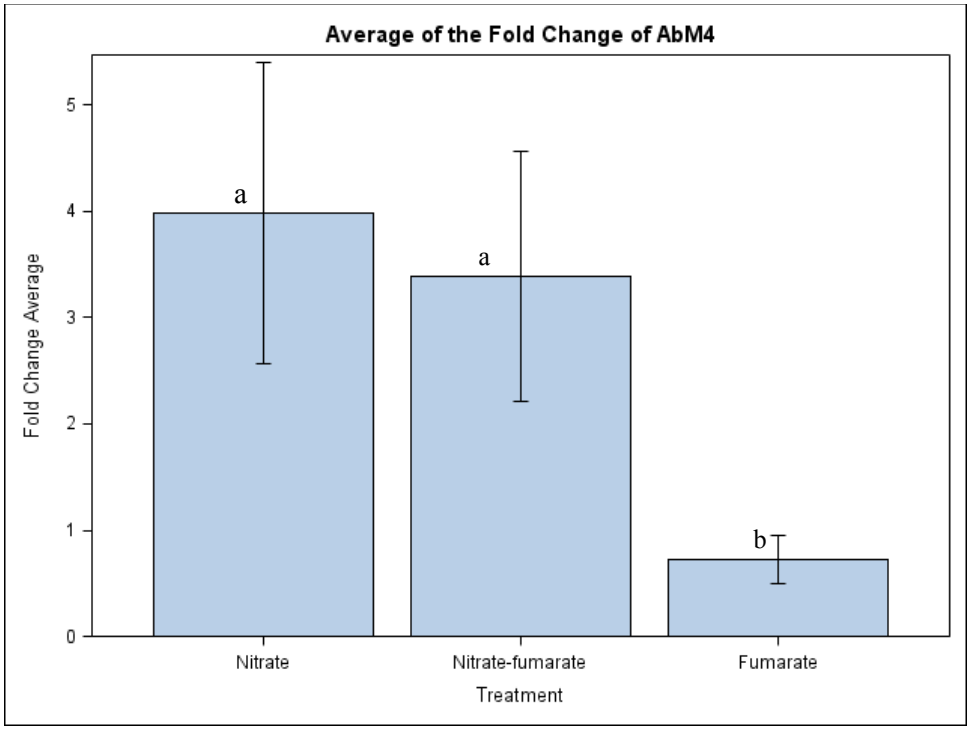

Figure 7. The Relative Abundance of *Methanobrevibacter sp. AbM4*.

Figure 8 shows the effect of feed additives on the fold change in DNA for *Methanosphaera stadtmanae*. The addition of fumarate increased ($p<0.05$) the relative

abundance of *Methanosphaera stadtmanae* compared to nitrate. Nitrate-fumarate addition also seemed to increase the relative abundance for M. *stadtmanae*, but differences observed were not significant different compared to nitrate or fumarate alone. Although the addition of the feed additives had no effect on total methanogen DNA, the addition of the feed additives to the diet seem to have a species-specific effect on M. *stadtmanae* and *Methanobrevibacter sp*. AbM4.

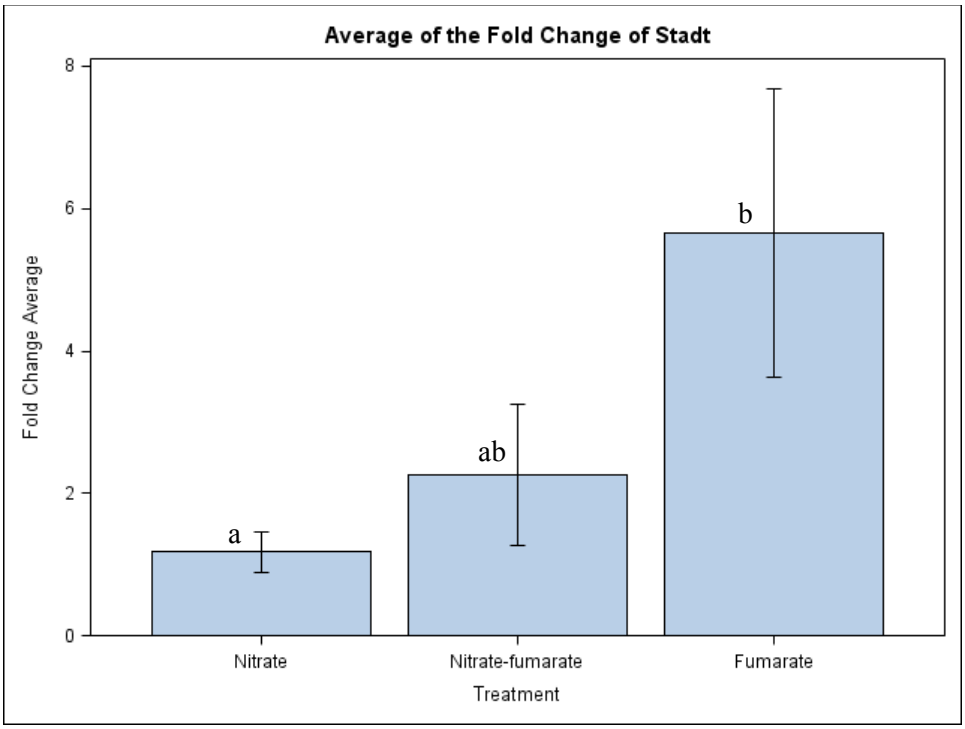

Figure 8. The Relative Abundance of *Methanosphaera stadtmanae*.

2.5 Discussion

2.5.1 Effects of Feed Additives on *in vitro* Methane Production. The fundamental principle behind the use of nitrate and fumarate alone or in combination was to take advantage of their ability to act as alternative hydrogen sinks and determine if the combination could be more effective in suppressing methane production. The addition of nitrate in the current study consistently reduced *in vitro* CH_4 production compared to the control or fumarate in both experiments. The reduction of CH_4 production is most likely due to the energetically more

favorable use of nitrate for its own reduction to nitrite and eventually to ammonia (Hulshof et al. 2012 and van Zijderveld et al. 2010). Our findings are in agreement with previous work by van Zijderveld et al. (2010), van Zijderveld et al. (2011) and Hulshof et al. (2012) who reported that nitrate consistently reduced *in vivo* CH_4 production in lambs, Holstein-Friesian cows, and beef cattle by as much as 32, 16, and 32%, respectively, while Zhou et al. (2011) reported that the addition of nitrate decreased CH_4 production by as much as 70%. The decrease in CH_4 production in the current study could also be due to an increase in the number of nitrate-reducing bacteria (Iwamoto et al. 2002) that obtain energy from nitrate/nitrite reduction making them formidable competitors to methanogens for H_2 consumption, that may lead to a decrease in the abundance of methanogens (Zhou et al. 2011) and in turn reduce enteric methanogenesis.

The current study also revealed that fumarate did not reduce CH_4 production. The lack of inhibition by fumarate on CH_4 production was unexpected because the reduction of fumarate to propionate is more exergonic than methanogenesis (Ungerfeld et al. 2007) and also the stimulation of fumarate-utilizing bacteria which can compete with methanogens for H_2 (Asanuma et al. 1999). Also the addition of fumarate has been shown to increase propionate production (Asanuma et al. 1999) and propionogenesis is another H_2 utilizing process that can compete against methane production (Boadi et al. 2004 and Asanuma et al. 1999). The addition of disodium fumarate has been shown to increase the abundance of *Selenomonas ruminantium,* a fumarate reducing bacteria and reduce methanogens in goats (Yang et al. 2012). However, the observations in the current study were similar to an earlier report by Beauchemin and McGinn (2006a and 2006b) who found no effect of fumarate on total daily CH_4 emissions in beef cattle. The efficacy of fumarate *in vitro* has also been linked to the ruminant animal's diet, for instance a study by Carro and Ranilla (2003) indicated that CH_4 reduction by the addition of fumarate was

successful when the ruminant animal was on a concentrate based diet and CH_4 production remained constant in the absence of the concentrate diet. The addition of fumarate to reduce CH_4 production being diet dependent is also supported by Castro-Montoya et al. (2012) who observed that the inhibition of *in vitro* CH_4 production was greatest in concentrate samples than in grass silage samples. On the other hand, Lopez et al. (1999) and Asanuma et al. (1999) reported only small decreases, 5% to 11% in CH_4 production by the addition of fumarate. The inability of fumarate to reduce CH_4 production can be diet-dependent since it has been reported that the utilization of fumarate by microorganisms is affected *in vitro* and *in vivo* by diet composition (Carro and Ranilla, 2003; Beauchemin and McGinn, 2006; Castro-Montoya et al. 2012) and also due to the incomplete conversion of fumarate to propionate (Ungerfeld et al. 2007) may have lead to an non-competitive action with methanogenesis.

The combination of nitrate and fumarate in the current study also reduced *in vitro* CH_4 production by 40 and 68% in experiments 1 and 2, respectively which was in agreement with van Zijderveld et al. (2010) who examined the combined effect of nitrate and sulfate on CH_4 production and concluded there was a 47% decrease in CH_4 concentrations when compared to nitrate (32%) and sulfate (16%) alone. In the current study when the combination of nitrate-fumarate was compared to nitrate there was no significant difference in experiment 1, although experiment 2 revealed that the greatest reduction in CH_4 production resulted from the use of the nitrate-fumarate combination. Iwamoto et al. (2002) reported that fumarate may serve as energy and carbon source for growth for nitrate-reducing bacteria, therefore assuming this then the stimulation of the fumarate and the nitrate reducing bacteria by the addition of fumarate should have a greater reduction in methanogenesis. The coupling of fumarate with other strategies was proposed by Adbl-Rahman et al. (2010) who revealed that fumarate coupled to a defaunating

agent was successful in reducing *in vitro* CH_4 production, while fumarate alone had no effect on *in vitro* CH_4 production. The reduction in CH_4 production in the current study by the addition of nitrate-fumarate combination compliments the study by Iwamoto et al. (1999) who reported that nitrate is rapidly converted to nitrite and if accumulated in the rumen it may inhibit fermentation. Therefore increasing the rate of nitrate/nitrite reduction by using of alternate feed additives like fumarate can alleviate this undesirable effect on nitrate. Iwamoto et al. (1999) reported that 20 mM of nitrate completely inhibited CH_4 production suggesting fermentation was suppressed on the other hand, the addition of fumarate at 15 and 30 mM with nitrate (10 mM) decreased CH_4 production and increased the rate of reduction of nitrate, but the study also revealed the mixture was not as successful in reducing CH_4 production as nitrate alone. Experiment 2 in the current study revealed that the mixture of nitrate-fumarate was at least as effective as or greater than nitrate at inhibiting CH_4 production. This could be due to the fact that we added nitrate and fumarate as a 1:1 ratio whereas Iwamoto et al. (1999) increased the fumarate to nitrate ratio to 1: 1.5 and 1:3 and the reduction of fumarate to methane precursors like acetate could have influenced methanogenesis. The results from the current study also revealed that when adding nitrate and fumarate in a 1:1 ratio may be more effective at reducing CH_4 production than the use of nitrate or fumarate alone.

Rumen contents are very heterogeneous physically and microbiologically (Zebeli et al., 2008). Therefore microorganisms that inhabit various locations in the rumen including particulate and fluid fractions of rumen fluid should have an impact on rumen fermentation and possibly gas production (Zebeli et al., 2008). In the current study the PS effect on CH_4 production varied across both experiments. Although the medium PS was consistently lower than the large PS in both experiments, the decrease in CH_4 production for the small PS revealed there

was no obvious trend in the effect of PS on CH_4 production. This could be due to the fact that the particle sizes selected for the study was contiguous increments making the PS effect on CH_4 production less clearly defined. However a study by Zebeli et al. (2008) revealed that PS of grass hay at 6 mm and 30 mm had no effect on gas production.

2.5.2 Effect of Feed Additives on Total Microbial DNA. The current study did not find any effect of feed additives on total microbial DNA extracted from the *in vitro* fermentation samples. According to the literature there are different views about the usefulness of the DNA extraction kit, QIAamp DNA Stool Mini kit (Li et al., 2003). However despite all the inconsistencies about selecting the appropriate DNA extraction technique for gut microbial studies the kit was used in the current study. Henderson et al. (2013) indicated that not all DNA extraction techniques are suitable for diverse microbial groups and can have an impact on the representation of the microbial communities. The current location of sampling within the rumen may have impacted the microbial diversity represented within samples based on reports by Shin et al., (2004) and Janssen & Kirs, (2008). The observation that there was no effect of feed additives on total microbial DNA could be due to the fact that rumen contents were primarily fluid fractions resulting in a sample less representative of particulate associated microorganisms. DNA extraction techniques can also influence the representation of methanogens in samples, for instance, Henderson et al. (2013) reported there was a lower relative abundance of *Methanosphaera stadtmanae* using the QIAamp DNA stool kit than the bead beating methods suggesting that the *Methanosphaera* group requires greater disruption to release DNA, while *Methanobrevibacter ruminantium* clade abundance was consistent for the bead beating and chemical lysis step of the QIAamp DNA stool kit (Henderson et al., 2013).

2.5.3 Effects of Feed Additives on the Abundance of Methanogens. Rumen modifiers like nitrate and fumarate have long been shown to alter rumen microbial populations (Zhou et al., 2011) and play a major role in the dynamics of the rumen all the way down to methanogens (Ozutsumi et al., 2006).The current study investigated the effects of nitrate, fumarate and nitrate-fumarate mixture on the relative abundance of total methanogens and *Methanobrevibacter sp. AbM4*, and *Methanosphaera stadtmanae* species. Methanogen populations have been evaluated in response to dietary treatments and it has been reported that nitrate and chloroform reduce the abundance of methanogens (Zhou et al., 2009; Knight et al., 2011). The results from the current study revealed that total methanogens were not affected by the addition of the feed additives. This finding was in agreement with Mohammed et al. (2011) who observed dry corn grains and condensed tannins had no effect on total methanogens. A study published by Zhou et al. (2011) also revealed that Methanobrevibacter sp. AbM4 was detected for the first time in the bovine rumen however the study also revealed that the feed efficiency of the animal had no effect on total methanogen populations, but feed efficiency had an effect on M. *stadtmanae* and *Methanobrevibacter sp.* AbM4.

In total contradiction to our findings Zhou et al. (2011) revealed that sodium nitrate (12 mM) decreased total methanogen populations. Our results show that the addition of nitrate, fumarate and nitrate-fumarate combination increased the relative abundance of *Methanosphaera stadtmanae* and *Methanobrevibacter sp. AbM4*. Methanogens can have a preference to use H_2 as a substrate for energy and growth (Hook et al., 2010). *Methanobrevibacter ruminantium* and *Methanobrevibacter spp*. are species that can use H_2 and CO_2 as substrates for methanogenesis. Also M. *stadtmanae* and *Methanobrevibacter sp*. AbM4 are both hydrogenotrophic and can use H_2 as a substrate for methanogenesis (Leahy et al. 2013).

Since it is already known that *Methanosphaera stadtmanae* prefers H_2 but can also use methanol for methanogenesis then the lower relative abundance of M. *stadtmanae* observed by the addition of nitrate compared to the abundance observed by the addition of fumarate could possibly be related to the effect of nitrate consuming H_2 for its own reduction and as result has an indirect effect on *Methanosphaera stadtmanae* abundances. In the current study *Methanosphaera stadtmanae* responses to fumarate could be related to the dependence on acetate for biosynthesis of cell components (Fricke et al., 2006). The fold change in DNA for *M. stadtmanae* was greatest for fumarate and that could be due to the fact that fumarate may have been synthesized to acetate. Lopez et al. (1999) also reported that sodium fumarate had no effect on the quantity of methanogens, which may explain why fumarate did not affect total methanogens.

The rumen environment can change due to feed additives (Kluber & Conrad, 1998; Knight et al., 2011). The use of nitrate is shown to reduce methanogenesis, but the effect on methanogens could be species related (Kluber & Conrad, 1998) and has shown to have no effect on methanogens (Anderson & Rasmussen, 1998). The addition of nitrate had less impact on the relative abundance of *Methanobrevibacter sp. AbM4* indicating that nitrate was less effective on inhibiting this particular methanogen species. These findings are in agreement with the report by Zhou et al., (2012) where fumarate was effective in inhibiting the growth of methanogens. The decrease in the abundance of *Methanobrevibacter* sp. AbM4 could also be due to the fact that fumarate increases fumarate-reducing bacteria that are known to compete with methanogens for H_2 (Zhou et al., 2012). The results from the current study further indicate that these two methanogenic species behave differently in the presence of nitrate and fumarate. The complete genome of *Methanobrevibacter sp*. AbM4 (Leahy et al., 2013) reveals that Methanobrevibacter sp. AbM4 is similar to *Methanobrevibacter ruminantium* M1 and that AbM4 could use CO_2, H_2,

and formate for methanogenesis and may occupy a ruminal niche different from *Methanobrevibacter ruminantium* M1 suggesting AbM4 is a methanogen that occupies the fluid portion of the rumen.

The addition of nitrate and nitrate-fumarate combination effectively reduced CH_4 production, but it is unclear if the reduction of CH_4 production was due to reduction of VFAs or due to possible changes on the microbial populations in the rumen. The reduction in VFA may indicate a reduction in microbial fermentation with the implication that if similar effects were to be observed *in vivo* animal performance could possibly be compromised. The addition of fumarate may not seem to be useful in the reduction of CH_4 as a single dose. There was no effect of feed additives on the total microbial DNA or the relative abundances of total methanogens from the *in vitro* samples. It was interesting that *Methanobrevibacter sp. AbM4* and *Methanosphaera stadtmanae* relative abundances were different in the presence of nitrate and fumarate. Suggesting substrate availability and preference may have played a role in their abundance where in the presence of nitrate *Methanobrevibacter sp. AbM4* can better adapt to environments that have less free H_2. Therefore, the use of nitrate alone or in combination with fumarate can be useful feed additives to reduce CH_4 production. The optimal dose for nitrate as a feed additive needs further investigation to lessen the fear for nitrate toxicity, its impact on VFA production and fermentation, while the use of nitrate-fumarate seems to effectively reduce CH_4 production without the adverse effects of nitrate if used alone.

2.6 Conclusion

In conclusion based on the results the addition of nitrate alone or in combination with fumarate can effectively reduce *in vitro* methane production. The addition of fumarate was not effective in reducing methane production therefore nitrate in the combination of these feed

additives is responsible for the decrease in methane production. Likewise the lack of influence from the addition of these feed additives on total methanogens suggests that the feed additives created an environment that favored nitrate reducing bacteria and they effectively competed for free hydrogen for the reduction of nitrate to be used for their own energy and growth. Since fumarate can be reduced to acetate and M. *stadtmanae* can use acetate for biosynthesis then *Methanosphaera stadtmanae* can better adapt to the addition of fumarate. Also *Methanobrevibacter sp. AbM4* is a species that is known to thrive in low hydrogen environments and the species can better adapt to the addition of nitrate. Also reducing the particle size of the feed in the current study did not reveal any consistent pattern on methane production indicating that particle size differences used in the study were not large enough to understand the effect of feed particle size on methane production. The observed decreases in methane production did not correlate with the increases in the relative abundances of the two methanogen species indicating their contribution to the methanogen population and methane production was small or not significant. Therefore, the current study demonstrated that nitrate alone or in combination with fumarate can favor a reduction of *in vitro* methanogenesis.

CHAPTER 3

Influences of Feed Particle Size and Feed Additives on *in vitro* VFA Production

Abstract

This study investigated the effects of feed particle size and nitrate or fumarate alone or in combination on *in vitro* VFA production. Rumen fluid was collected from a Holstein-Friesian dry cow and steer (experiment 1) and dry cow and two steers (experiment 2) averaging 625.4 kg in BW. Animals were offered 11.4 kg/d per animal of concentrate diet containing equal amounts of soybean meal, whole cottonseed, and ground corn once a day with free access to hay (experiment 1) and 15.9 kg/d per animal of TMR consisting of silage, hay, corn, corn gluten, soybean meal, and minerals (experiment 2). Feed grab samples were collected, oven-dried, coarsely pulverized in a regular kitchen blender for one minute, and separated into three particle sizes (PS: 0.85, 1.4 and 2.36mm). The feed additives used were nitrate, fumarate and a nitrate-fumarate combination. Concentrations of VFAs were measured by Gas Chromatography. The results of the study revealed that nitrate decreased ($p < 0.05$) individual VFA production compared to the control for both experiments. The current study also showed a 46% decrease total VFA production by the addition of nitrate compared to the control. On the other hand, fumarate had no effect on individual and total VFA production in experiment 1, but fumarate increased ($p < 0.05$) production of propionate and reduced ($p < 0.05$) butyrate, valerate, and iso-valerate compared to the control in experiment 2. The addition of fumarate in experiment 2 had no effect on acetate or iso-butyrate production. In experiment 1, the addition of the nitrate-fumarate combination decreased ($p < 0.05$) butyrate and iso-butyrate production compared to the control and had no effect on all other individual VFAs. In experiment 2 the addition of nitrate-fumarate also decreased ($p < 0.05$) acetate, butyrate, valerate, and iso-valerate, but had no effect

on propionate and iso-butyrate concentrations compared to the control. There was no effect of PS observed in experiment 1. However, in experiment 2, the concentrations of acetate, butyrate, and propionate decreased as PS increased ($p < 0.05$). There was no effect of PS on iso-butyrate and iso-valerate concentrations in experiment 2. The addition of nitrate also increased ($p < 0.05$) the acetate to propionate ratio compared to the control, fumarate or nitrate-fumarate combination for both experiments. The addition of fumarate and nitrate-fumarate had no effect on the acetate to propionate ratio compared to the control. The addition of nitrate alone could possibly have an adverse effect on microbial fermentation if VFA production is significantly decreased. The addition of fumarate had no adverse effect on VFA production. Therefore, the addition of the nitrate-fumarate combination could possibly be used as a viable feed additive to shift VFA production and reduce the availability of H_2 for CH_4 synthesis.

Keywords: *in vitro*, *VFA, nitrate, fumarate, particle size*

3.1 Introduction

Dietary manipulation can have profound effects on the concentrations of VFA in the rumen. Boadi et al. (2004) reported that there is a direct relationship between VFA concentration and CH_4 production with acetate, propionate and butyrate noted as the three major VFAs produced in the rumen. Microorganisms in the rumen are responsible for the fermentation of carbohydrates in the diet to VFAs, which the animal uses as energy sources (Boadi et al., 2004). The major VFAs that are produced during fermentation have different functions in the animal (Boadi et al., 2004). While the non-glucogenic fatty acids (acetate and butyrate) are used by the animal for milk fat and long-chain fatty acid synthesis, propionate on the other hand is used for glucose synthesis (Morvay, Bannink, France, Kebreab, & Dijkstra, 2011). The production of fermentation end products like VFAs leads to free hydrogen (H_2) to be utilized in

the rumen for various processes. Since there is a direct relationship between VFA and CH_4 production it is important to explore how dietary manipulation can shift VFA production and reduces the availability of H_2 for methanogenesis. The addition of organic acids like fumarate and malate has been shown to shift VFA profiles in favor of propionate, and prevent the availability of H_2 in the rumen for CH_4 synthesis (Boadi et al., 2004).

Dicarboxylic acids including fumarate have long been proposed as rumen modifiers by enhancing the succinate-propionate pathway (Araújo et al., 2011) and is a key intermediate product in rumen microbial metabolism (Yu et al., 2010). There are several studies (Mao et al., 2008; Abdl-Rahman et al., 2010; Wood et al., 2009; Yu et al., 2010) that have focused on using fumarate to stimulate propionate production. Fumarate may also act as an electron acceptor and reduce the availability of H_2 and also increases acetate production in the rumen (Ungerfeld et al., 2007). Nitrate and other nitro-compounds have also been investigated as feed additives *in vitro* (Anderson & Rasmussen, 1998; Bozic et al., 2009) and *in vivo* (Hulshof et al., 2012; van Zidjerveld et al., 2010) trials to reduce CH_4 production. Nitrate has been shown to increase the production of acetate while decreasing propionate production (Farra & Satter, 1971 Bozic et al., 2009; Zhou et al., 2011), however at high concentrations nitrate had no effect on acetate production (Zhou et al., 2011). The use of nitrate in the ruminant diet also raises the risk of methemoglobinemia unless the ruminant is allowed to adapt to nitrate by slowly introducing it to the diet (van Zidjerveld et al., 2010). Normally, fumarate is converted to succinate and then to propionate, while nitrate is reduced to nitrite that leads to the production of ammonia in the rumen and both reactions can reduce the availability of H_2 for CH_4 synthesis. Fumarate consumes H_2 for propionogenesis while nitrate consumes H_2 for its own reduction to NH_3. The risk of nitrate toxicity has led to the investigation of combining feed additives with nitrate to reduce this

undesirable effect by increasing the rate of NO_3 disappearance *in vivo* (Sar et al., 2004) and in vitro (Anderson & Rasmussen, 1998; Iwamoto et al., 1999). Iwamoto et al. (1999) reported that addition of fumarate to nitrate increased the rate of nitrate and nitrite reductions while propionate and acetate production increased. The additive effect of fumarate and nitrate on *in vitro* VFA production is not yet clearly understood.

It has also been reported that a reduction in feed particle size (PS) increases microbial attachment resulting in increased digestion and production of VFA (Bhandari et al., 2008) while a reduction in PS *in vivo* could mean an increase in the rate of passage and exit from the rumen (Janssen, 2010). Mastication reduces particle size of ingested feed and is necessary for consumption of feed. This primary process in digestion is thought to have an impact on rumen function because the feed is manipulated before being fed to the animal. The particle size of the feed may also affect the rumen environment and the resident bacteria. Kononoff et al. (2003) evaluated the effect of different particle sizes of corn silage *in vivo* on chewing activities and rumen fermentation in lactating Holstein cows. The study revealed that concentration of total VFAs increased linearly as particle size of the silage decreased. The effect of forage PS on pH and rumen fermentation has also been evaluated in lactating Holstein cows by Bhandari et al. (2008) and they concluded that reducing the PS of alfalfa and oat forage increased DMI, while concentrations of total VFAs and acetate to propionate ratios were not affected in both forages.

In vitro rumen fermentation techniques provide knowledge on the fermentation process and how the different feed additives may alter conditions that can affect digestion (Castro-Montoya et al., 2012). Although *in vitro* fermentation methods are not substitutes for *in vivo* rumen fermentation they can and have been used extensively to elucidate the basic biochemical processes performed by rumen microorganisms and the factors that affect them. It is evident that

fumarate increases propionate concentrations, while nitrate reduces individual VFA concentrations indicating a suppression of fermentation. Therefore it was hypothesized that a nitrate-fumarate combination would increase propionate and moderately decreases VFA concentrations without negatively impacting *in vitro* fermentation, while feed particle will influence VFA production. The current study was conducted to quantitatively and qualitatively determine the effects by the addition of nitrate, fumarate and nitrate-fumarate combination with feed PS on *in vitro* VFA production.

3.2 Materials and Methods

3.2.1 Animals, Sampling Methods, and *in vitro* Techniques Used in the Study. The experiment was approved by the North Carolina Agriculture & Technical State University Institutional Review Board. Two experiments were completed to investigate the effect of nitrate, fumarate , and nitrate-fumarate with three different particle sizes of feed large, medium, and small (2.36, 1.4, and 0.850 mm) on the fermentation end products VFAs. Experiment 1 consisted of two Holstein-Friesian cattle (steer $n = 1$; dry cow $n = 1$) average BW = 650 kg fed a daily feed allowance of 11.4 kg/d per animal of equal amounts of soybean meal, whole cottonseed, and ground corn once a day and offered free access to grass hay when grass was not available in the winter months and allowed to graze on forage during the spring. Experiment 2 consisted of three dry Holstein-Friesian animals (dry cow $n = 1$; and steers $n = 2$) average BW = 624.1kg fed 15.9 kg/ day per animal TMR of silage, hay, corn, corn gluten, soybean meal, and minerals supplements. All diets were formulated to meet the nutrient requirements of all animals.

Rumen contents were obtained from the rumen (Bar Diamond, Idaho, US) at 09.00 h prior to feeding. The experiments were conducted from January 2012-August 2012 and January 2013- August 2013. Rumen digesta from previous day were removed to thoroughly mix rumen

contents. The mixed rumen contents were removed and strained through eight layers of cheesecloth into a pre-warmed thermos to a volume (1L) to minimize oxygen in the headspace and primed with CO_2; sealed tightly and transported to the university laboratory for analysis. Prior to start of the experiment feed samples for the experiment were collected from the feed trough and oven dried at 70°C for 24 h for lab analysis. Feed grab samples was coarsely pulverized in a kitchen blender for one minute and separated into three particle sizes (PS)(2.36, L; 1.4, M; and 0.85, S) mm using standard test sieves (Fisher Scientific Company, US). The in vitro method for the determination of VFA production was carried out according to the first stage *in vitro* digestibility procedure of Tilley and Terry (1963). The artificial saliva was prepared overnight at 39°C according to the procedures McDougall (1948).

3.2.2 Chemical Analyses of Feed. Table 10 shows the chemical composition of feed for experiments 1 and 2. Chemical composition was determined after samples were oven dried 70°C for 24 h. Dry matter (DM) was determined in duplicate by weighing 2.0 g of feed into pre-dried crucibles and dried in the oven at 100°C overnight, crucibles were allowed to cool in a desiccators, and weighed (Ohaus Explorer Pro balance, Parsippany, NJ, USA). The ash content was determined by placing the feed samples in crucibles from the DM experiment into muffle-furnace at 550°C for one hour and weighed and percent ash was calculated. Neutral detergent fiber (NDF) using α-amylase and sodium sulfite and acid detergent fiber analysis(ADF) was also determined according to Van Soest (1963) by grinding the feed samples to 1mm and 0.50 g of the air dry feed was placed into F57 fiber filter bags (ANKOM Technology, USA) and heat sealed. The extraction method for determining neutral and acid detergent fiber provided by ANKOM Technology was followed to complete the process. After the end of the procedure the sample bags were placed in the oven at 70°C overnight and weighed using the Ohaus Explorer

Pro balance (Parrsippany, NJ, USA). Crude Protein analysis of feed was determined on the TruSpec CN (Leco Corporation, Michigan, US). Feed samples (0.1g) were placed in foil cups from Leco Corporation (Michigan, US) and analyzed for protein in triplicate with EDTA as the standard and carrier gases were compressed air, Helium, and Oxygen. The procedure for sample preparation and analysis was followed as recommended by the manufacturer.

Table 10

Chemical Composition of Experimental Diet by Particle Size

	Experiment 1			Experiment 2		
Particle size , mm	**Large**	**Medium**	**Small**	**Large**	**Medium**	**Small**
Dry matter, %	94.71	89.52	90.90	89.62	91.08	89.92
Total Ash, %	12.5	3.13	12.4	3.13	3.13	3.10
Crude protein[a], %	12.38	-	-	11.98	-	-
aNDF %	56.28	46.28	26.73	54.86	44.59	26.23
ADF%	29.68	25.16	13.77	33.95	26.86	14.15
Ca, ppm	23.4	33.2	37.8	17.9	19.6	38.4
K, ppm	46.3	45.6	33.6	50.4	39.3	42.0
Mg, ppm	10.0	10.1	8.6	12.2	9.7	9.6
P, ppm	23.0	28.2	18.8	22.4	19.8	20.9

[a] representative of total mixed ration before separated into particle size; NDF (neutral detergent fiber); ADF (acid detergent fiber); Ca (calcium), K (Potassium), Mg (Magnesium), P (Phosphorus)

3.2.3 Determination of VFA Concentrations. Sample preparation included collecting 30ml aliquots of ruminal fluid from *in vitro* fermentation flasks into 50 ml conical tubes, centrifuged at 4000 rpm for 15 minutes at 4°C and frozen at -20°C until analysis. A 5 mL aliquots of rumen fluid from each conical vial was transferred by pipette into a 15 mL centrifuge tube and 1 mL of a 25% aqueous meta-phosphoric acid was added and vortexed to precipitate the proteins (Cottyn and Boucque, 1968). The mixture was allowed to stand for 30 minutes and centrifuged at 3500

rpm for 20 min at 15°C. Approximately 1 mL of the mixture from each tube was carefully transferred to GC vials for analysis. Standards for the VFAs were prepared using reagent grade acids (Sigma-Aldrich, St. Louis, US). Standard aliquots of 0.50 mL were transferred into 10 mL vial and brought up to a volume of 3.0 mL and the weight of each acid was determined using the specific gravity of the acid and used to determine the ppm (mg/L) of each acid. The VFA profiles and concentrations of acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate were determined on the Thermo Fisher Trace Ultra gas chromatograph coupled to a flame ionization detector with a Tri-Plus auto sampler. The GC specifications were: Column: Nukol, 15 m, 0.53 mm id, 0.50 um film thickness, Injector and detector temperature: 210°C, Carrier: Helium, constant flow 1.0 ml/minute, Gas flows: Air 350 ml/minute, Hydrogen 35 ml/minute, and Nitrogen 30 ml/minute, Oven parameters: start 70°C hold 4 minutes, 70°C at 6°C/min to 200°C hold 1 minute. The VFA concentrations were identified from their retention time of the standards and quantified from measurement peak areas (see Figure 9).

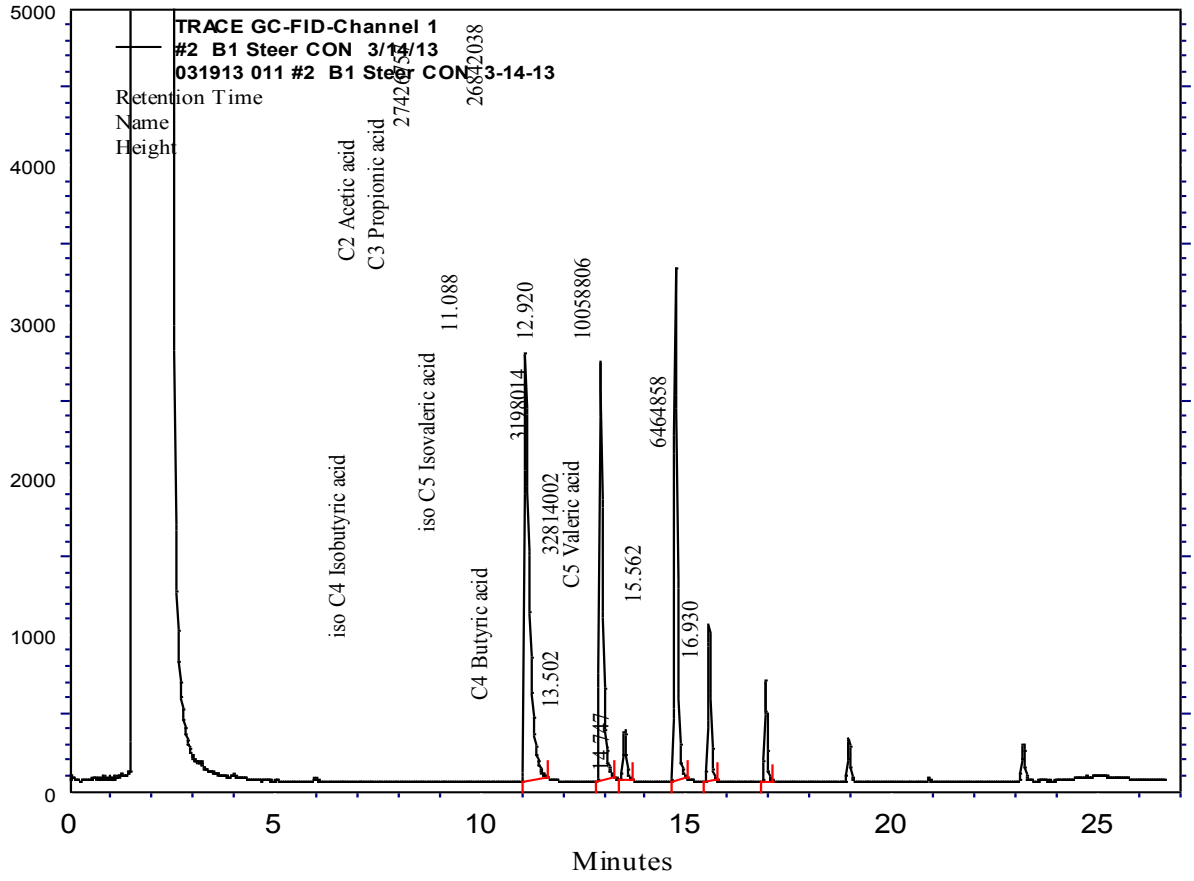

Figure 9. The Elution Sequence and Separation of Volatile Fatty Acids Determined by Gas Chromatography.

3.3 Experimental Method

The experiment was conducted using three PS (L, 2.36; M, 1.4; and S, 0.850 mm) of feed and three feed additives as treatments nitrate, fumarate, and nitrate-fumarate and a control on *in vitro* VFA production.

3.4 Statistical Analysis

Data for the *in vitro* VFA production was analyzed using SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). The analysis was conducted in a randomized complete block design with animals as blocks, and 3 replications of the particle size and feed additive treatments per block. Volatile fatty acid production data was analyzed with PROC MIXED of SAS, using a

mixed model with animals as a random effect and particle size and feed additive treatments as fixed effects (Little, Milliken, Stroup, Wolfinger, & Schabenberger, 2006; SAS Institute, 2012). The particle size by feed additive interaction and the fumarate by nitrate interaction were examined and where these interactions were not significant sample treatments were compared independent of feed particle size and all treatment effects were declared significant at $p < 0.05$. All treatment effects $p > 0.06$ and $p < 0.10$ were declared trends.

3.5 Results

3.5.1 Effect of Feed Additives on VFA Production.

3.5.1.1 Experiment 1. Figure 10 shows the effect of feed additives on individual VFA production. The addition of nitrate reduced ($p < 0.05$) individual VFAs compared to the control, fumarate and nitrate-fumarate combination. The addition of fumarate and the nitrate-fumarate combination had no effect on acetate production compared to the control and were not statistically different when compared to each other. The addition of nitrate reduced ($p < 0.05$) propionate, while the addition of fumarate and nitrate-fumarate combination had no effect on propionate production compared to the control. The addition of nitrate and the nitrate-fumarate combination also reduced ($p < 0.05$) butyrate, while the addition of fumarate had no effect on butyrate production compared to the control. The addition of nitrate and the nitrate-fumarate combination decreased ($p < 0.05$) iso-butyrate compared to fumarate and the control. Nitrate and the nitrate-fumarate combination decreased ($p < 0.05$) valerate and iso-valerate production compared to the control. Fumarate had no effect on valerate and iso-valerate production. Therefore, while the addition of nitrate decreased all VFA production, addition of fumarate had no effect on individual VFA production compared to the control. However the nitrate-fumarate

combination numerically increased propionate production ($p < 0.10$). The addition of nitrate-fumarate also had no effect on acetate, valerate, and iso-valerate concentrations.

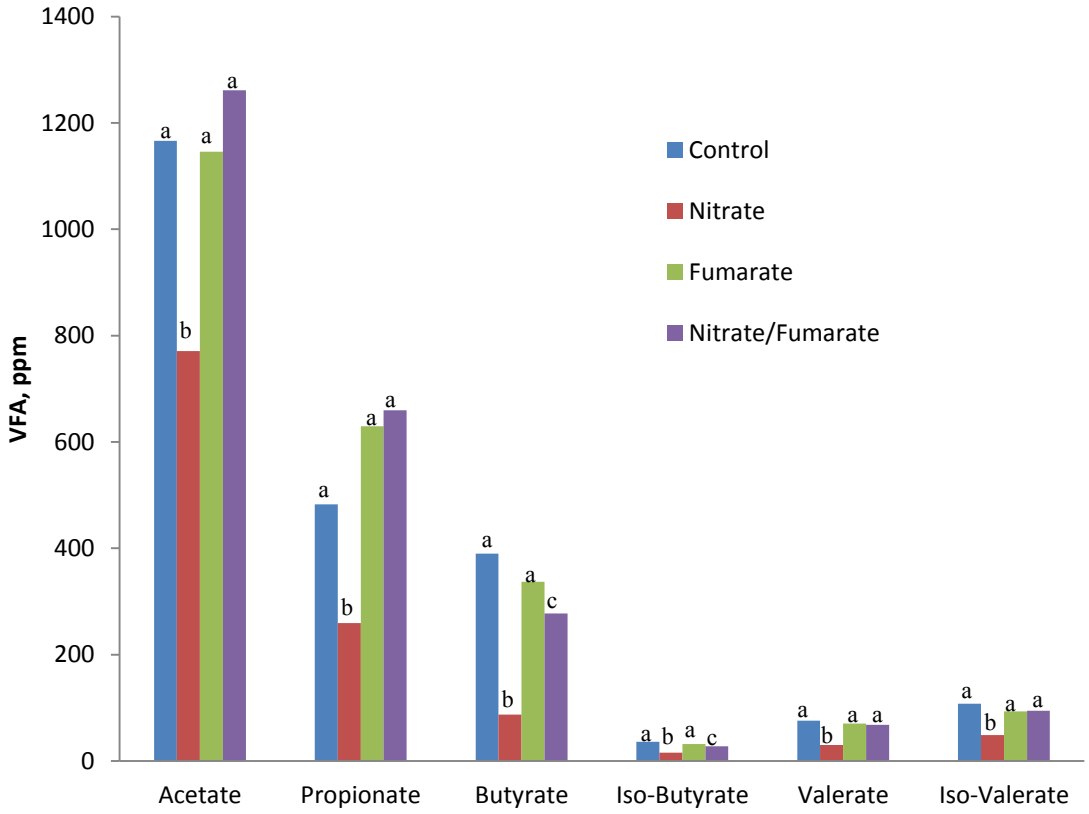

Figure 10. Effect of Feed Additives on Individual VFA Production (Exp. 1).

Bars with different superscripts within each VFA indicates means that are different ($p < 0.05$)

3.5.1.2 Experiment 2. Figure 11 shows the effect of feed additives on individual VFA production. In experiment 2, nitrate and the nitrate-fumarate combination decreased ($p < 0.05$) production of acetate compared to fumarate or the control. Also there was no significant difference between the reduction of acetate by nitrate and the nitrate-fumarate combination between the control and fumarate. The addition of nitrate decreased, while fumarate increased ($p < 0.05$) production of propionate compared to the control and nitrate-fumarate combination. Meanwhile, addition of the nitrate-fumarate combination had no effect on production of

propionate. The addition of nitrate, fumarate, and the nitrate-fumarate combination decreased concentrations of butyrate compared to the control and there were differences among the feed additives. The addition of nitrate also decreased ($P < 0.05$) iso-butyrate compared to the control, fumarate and nitrate-fumarate combination. Meanwhile the addition of nitrate, fumarate and nitrate-fumarate combination all reduced ($p < 0.05$) concentrations of valerate compared to the control, but there was no significant difference between nitrate and the nitrate-fumarate combination, while there were significant differences between fumarate and nitrate alone or in combination with fumarate. The addition of nitrate, fumarate and the nitrate-fumarate combination reduced ($p < 0.05$) iso-valerate concentrations compared to the control however the reduction between the feed additives was also significantly different. The addition of the nitrate-fumarate combination also decreased ($p < 0.05$) concentrations of acetate; butyrate, valerate and iso-valerate, but had no effect on propionate and iso-butyrate compared to the control. In general the addition of nitrate decreased the production of all individual VFAs. The addition of fumarate had no effect on acetate and increased the production of fumarate. Also the addition of fumarate decreased the production of butyrate, valerate and iso-valerate. The addition of the nitrate-fumarate combination decreased acetate, butyrate, valerate and iso-valerate. However addition of the nitrate-fumarate combination had no effect on the production of propionate and iso-butyrate.

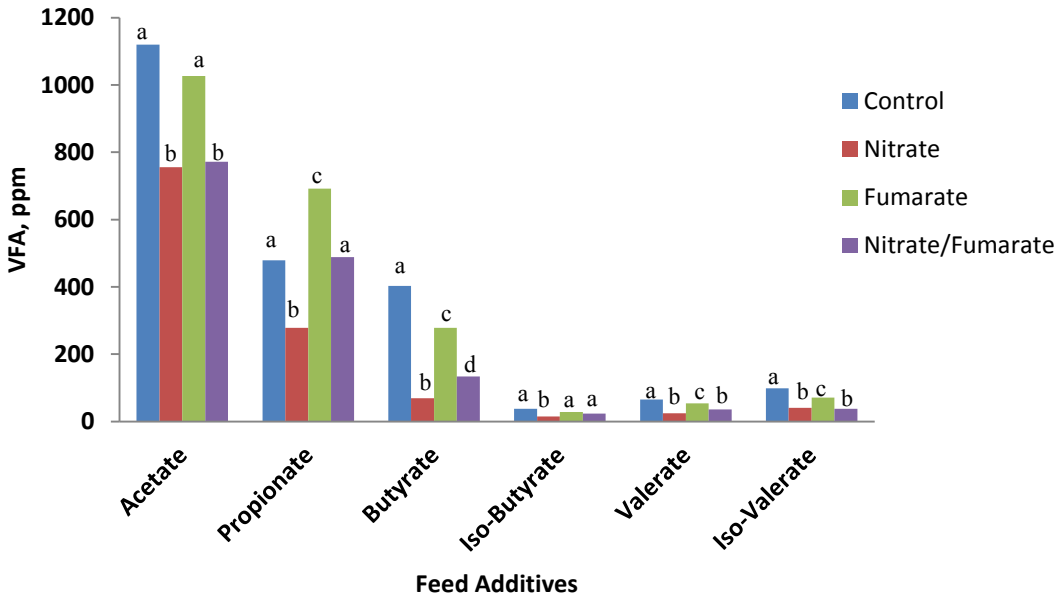

Figure 11. Effect of Feed Additives on Individual VFA Production (Exp. 2).

Bars with different superscripts within each VFA indicates means that are different ($p < 0.05$)

3.5.1.3 Effects of Feed Additives on the Acetate to Propionate Ratios. In experiment 1, the addition of nitrate increased ($p < 0.05$) acetate to propionate ratios compared to the control, fumarate and nitrate-fumarate combination (Table 11). The addition of fumarate and the nitrate-fumarate combination had no effect on the acetate to propionate ratio compared to the control. Table 11 also shows the effect of feed additives on acetate to propionate ratios in experiment 2. The addition of nitrate increased ($p < 0.05$) the acetate to propionate ratio compared to the control, fumarate and nitrate-fumarate combination. There was no significant difference between the addition of fumarate and the nitrate-fumarate combination for both experiments. Also the addition of fumarate and the nitrate-fumarate combination had no effect on the acetate to propionate ratio. Therefore the addition of nitrate increased the acetate to propionate ratios while the addition of fumarate and the nitrate-fumarate combination had no effect on the acetate to propionate ratios.

Table 11

Effect of Feed Additives on the Acetate to Propionate Ratios

		Control	Nitrate	Fumarate	Nitrate-Fumarate	SEM
Exp. 1	Acet: Prop Ratio	2.5^{a}	3.4^{b}	1.9^{a}	2.0^{a}	0.32
Exp. 2	Acet: Prop Ratio	2.4^{a}	5.7^{b}	1.5^{a}	1.6^{a}	0.99

Note: Different superscripts within a row indicates means that are different ($p < 0.05$)

3.5.2 Effect of Feed Particle Size on VFA Production.

3.5.2.1 Experiment 1. Figure 12 shows the effect of feed PS on individual VFA production. In experiment 1, there was no effect of PS on the production of acetate, propionate, butyrate, valerate and iso-valerate. However the production of iso-butyrate was higher ($p < 0.05$) for the large PS compared to the small and medium PS, but there was no effect of feed PS observed on all other VFAs.

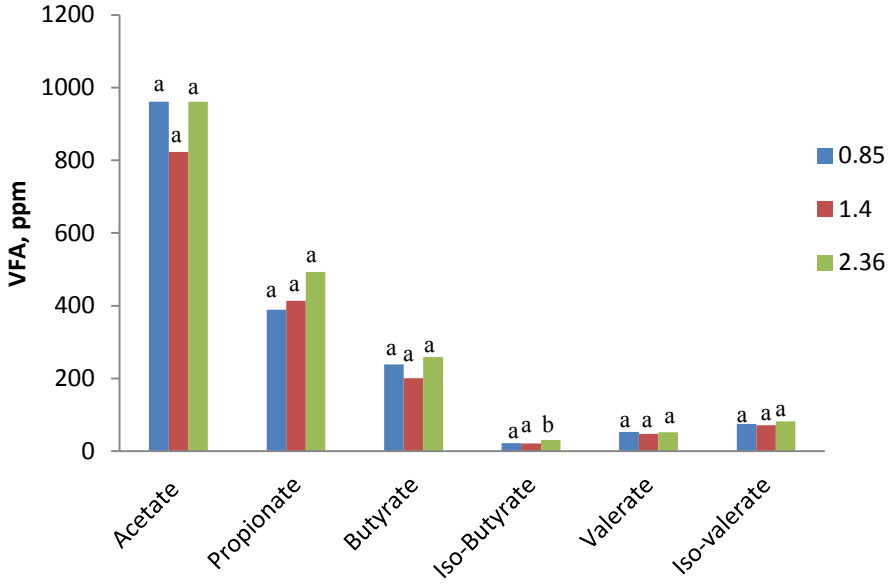

Figure 12. The Effect of Feed PS on Individual VFA Production (Exp. 1).

Bars with different superscripts indicate means that are different ($p < 0.05$)

3.5.2.2 Experiment 2. Figure13 shows the effect of TMR PS on individual VFA concentrations in experiment 2. The production of acetate decreased ($p < 0.05$) as the PS increased and there were significant differences among all PS. Also the production of propionate decreased ($p < 0.05$) as the PS increased and there were significant differences among the different PS. The production of butyrate also decreased ($p < 0.05$) as the PS increased, but there was no effect of PS on production of iso–butyrate. The production of valerate was lowest ($p < 0.05$) for the large PS compared to the small or medium PS and no significant difference was observed between small and medium PS. There was no effect of PS on the production of iso-valerate. The overall trend for individual VFAs namely acetate, butyrate and propionate in particular was that the concentrations decreased as PS increased ($p < 0.05$).There was no effect of PS on iso-butyrate and iso-valerate concentrations.

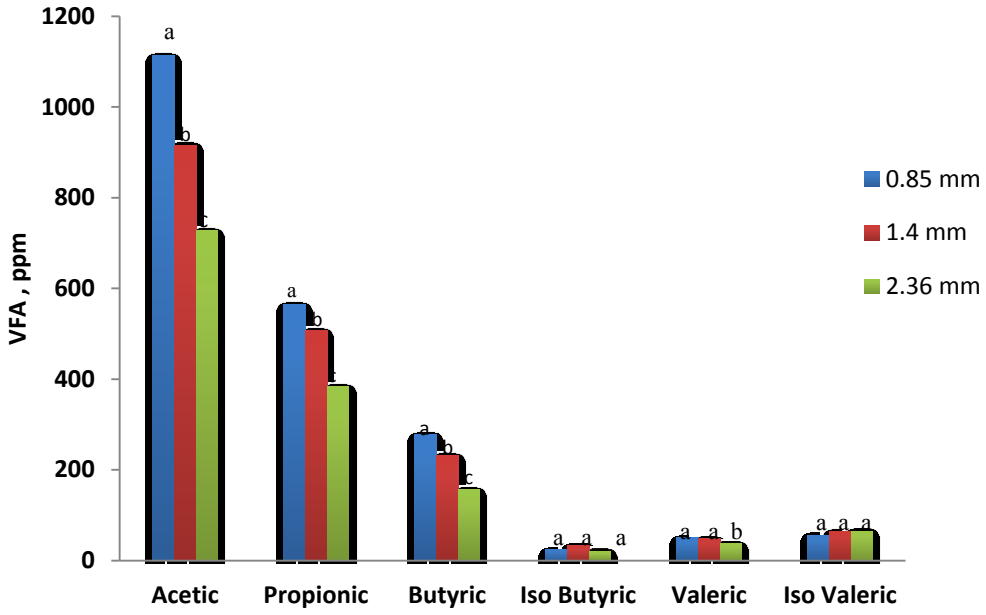

Figure 13. The Effect of Feed PS on Individual VFA Production (Exp. 2).

Bars with different superscripts indicates means that are different ($p < 0.05$)

3.5.2.3 Effects of Feed PS on the Acetate to Propionate Ratios. Table 12 shows the effect of feed PS on the acetate to propionate ratios for experiments 1 and 2. In experiment 1, the acetate to propionate ratio was observed to be higher ($p < 0.05$) for the medium PS compared to the small or large PS and the differences between the small and medium PS were not significant. Also in experiment 2 the acetate to propionate ratio was higher ($p < 0.05$) for the medium PS compared to the small and large PS. In general the medium PS seems to increase the acetate to propionate ratios but there was no significant difference between the small and large PS.

Table 12

Effect of PS on the Acetate to Propionate Ratios

	Acetate to Propionate Ratio			
Particle size, mm	**Small**	**Medium**	**Large**	**SEM**
Experiment 1	2.6[a]	3.0[b]	2.6[a]	0.14
Experiment 2	2.1[a]	4.2[b]	2.1[a]	0.71

Note. Different superscripts with in row indicates means that are significantly different ($p<0.05$)

3.6 Discussion

The main focus of this study was to investigate the individual and combined effects of nitrate and fumarate with feed particle sizes on *in vitro* VFA production. In the current study the addition of nitrate significantly reduced individual VFA production. The addition of nitrate also reduced total VFA production by 46% in both experiments, which is in agreement with Iwamoto et al. (1999) who reported that nitrate reduced total VFA production and this indicates that fermentation may possibly be suppressed. Also the decrease in total VFA production by the addition of nitrate observed in our study is in line with the report by Zhou et al., (2011) who indicated that the addition of nitrate decreased total *in vitro* VFA production. The addition of

fumarate in the current study had no effect on individual VFA production in experiment 1. The fact that the addition of fumarate had no effect on total VFA production in experiment 1 can be correlated to the observation that fumarate had no effect on individual VFA production in experiment 1 as well. The results in the current study revealed that fumarate had no effect on total VFA concentrations and the acetate to propionate ratio, which contradicts with reports from Bayaru et al. (2000); Carro and Ranilla (2003); Beauchemin and McGinn (2006a and 2006b); and Yu et al. (2010) who indicated that the addition of fumarate increased total VFAs concentrations. However, others (Abdl-Rahman et al., 2010; Mao et al., 2008) reported no effects of fumarate on total VFA concentrations. The addition of fumarate in the current study had no effect on acetate production in both experiments and this could possibly be explained by the fact that fumarate, even at low concentrations is thermodynamically favored to be reduced to acetate (Ungerfeld et al., 2007) and in our current study it could most likely be reduced by fumarate-reducing bacteria to succinate (Iwamoto et al., 1999) and then to propionate and acetate. However, fumarate increased *in vitro* propionate concentrations in the second experiment, which was in full agreement with Asanuma et al. (1999); Bayaru et al. (2000); and Yu et al. (2010). It was also observed in the current study that addition of nitrate increased the acetate to propionate ratios in experiments 1 and 2. This increased acetate to propionate ratio can best be explained by the fact that nitrate effectively reduced propionate by as much as 46% and 41% in experiments 1 and 2, and reduced acetate by only 34% and 33% in experiments 1 and 2, respectively. Therefore, although nitrate reduced acetate, reduction of propionate was to a much greater extent. The decrease in total VFA concentrations by the addition of nitrate in the present study could most likely be due to suppression of microbial fermentation (Iwamoto et al., 1999). On the other hand, fumarate has been shown to minimize the suppression of microbial

fermentation and enhance nitrate/nitrite reduction (Iwamoto et al., 1999) when combined with nitrate, which in effect reduces the adverse effects caused by nitrate on microbial fermentation. The combination of nitrate with fumarate in the present study showed variable effects on VFA production for example in experiment 1 only butyrate and iso-butyrate concentrations were reduced and had no effect on all other individual VFA. Although in experiment 2, the production of all VFAs was reduced by the addition of nitrate-fumarate combination except for propionate and iso-butyrate, however Iwamoto et al., (1999) reported that the addition of fumarate and nitrate increased propionate production. In experiment 1 the addition of nitrate-fumarate had no effect on total VFA production, but in experiment 2 the addition of nitrate-fumarate reduced total VFA production by 32%. This result contradicts with Iwamoto et al. (1999) who reported that nitrate and fumarate together increased total VFA production. However, in the current study we used a 1:1 nitrate to fumarate ratio where as Iwamoto et al. (1999) used 1:1.5 and 1:3 ratios. The addition of the nitrate-fumarate combination had no effect on the acetate to propionate ratios in both experiments. This result could possibly be due to the fact that the nitrate-fumarate combination had no effect on the production of acetate and propionate in experiment 1. Also in experiment 2, addition of the nitrate-fumarate combination reduced acetate, but had no effect on the production of propionate. Therefore, the results from both experiments could explain why the nitrate-fumarate combination had no effect on the acetate to propionate ratios. The effect of the addition of the nitrate-fumarate combination on VFA production in the current study shows that propionogenesis was not affected in both experiments. The fact that the addition of nitrate consistently reduced individual and total VFA may indicate that the addition of nitrate led to the accumulation of nitrite that inhibits microbial growth (Iwamoto et al., 2002 and Zhou et al., 2011). Nitrate reducing bacteria like *Selenomonas ruminantium*, *Veillonella parvula*, and

Wollinella succinogenes can also reduce fumarate (Iwamoto et al., 2002) as an energy source. Therefore, it is assumed that the addition of nitrate may have placed these bacteria in unfavorable conditions due to the accumulation of nitrite (Iwamoto et al., 1999) whereas the addition of fumarate could possibly provide these bacteria with sources of energy for growth.

In our study there was no effect of PS on VFA production in experiment 1. An earlier report by Bhandari et al. (2008) had indicated the lack of effect of PS on VFA production. Bhandari et al. (2008) who investigated the effect of forage chop length on rumen fermentation in Holstein cows revealed that VFA concentrations were not affected by the chop length of forages and also there was no effect on the acetate to propionate ratios. However, the results from the current study contradicts with Bhandari et al., (2008) and this could possibly be due to the fact that the current study was an *in vitro* experiment which measures VFA production, while *in vivo* studies on VFA production reflect a balance between production and absorption (Beauchemin and McGinn, 2006b). In experiment 2 it was observed that as particle size decreased acetate, propionate, and butyrate production increased. This finding was in agreement with Kononoff et al. (2003) who reported that total VFA concentrations increased as alfalfa haylage PS decreased in early lactation Holstein cows. The current study revealed that there was no effect on total VFA production in experiment 1. It was also reported by Storm & Kristensen (2010) that there was no effect of grass hay PS on VFA concentrations. However, in experiment 2 there was a 34% and 14% decrease in total VFA production for the small and medium PS. The reduction in feed PS increases the surface area of the feed and may in turn increase microbial fermentation. Therefore, the effect of PS on VFA from experiment 2 could be due to increased surface area for microbial attack and increase fermentation (Teimouri et al., 2004).

3.7 Conclusion

Reducing the particle size of the feed increased *in vitro* VFA production indicating that although the particle size increments were small, reducing the particle size may have increased the surface area for increased microbial fermentation. Consequently, the addition of nitrate consistently decreased individual and total VFA production suggesting that nitrate may have inhibited *in vitro* fermentation. The addition of fumarate did not exhibit any decreases on total VFA production, but increased propionogenesis instead. The combination of nitrate and fumarate could possibly be used as a potential feed additive to shift VFA production that would reduce the availability of precursors like acetate and butyrate for CH_4 synthesis, and increase the production of propionate. Therefore, combining fumarate and nitrate may be used to lessen the effects of nitrate on inhibiting VFA production. The current study suggests that combining fumarate with nitrate can reduce the undesirable effects of nitrate on VFA production while at the same time significantly reduce *in vitro* methane production.

CHAPTER 4

Influences of Feed Particle size and Feed Additives *in vitro* on pH, Nitrate, and Ammonia Production

Abstract

This study investigated the effects of feed particle size and nitrate or fumarate alone or in combination on *in vitro* pH, NO_3 and ammonia (NH_3) concentrations. The study was conducted in two separate experiments. Rumen fluid was collected from a Holstein-Friesian dry cow and a steer (experiment 1) and a dry cow and two steers (experiment 2) averaging 625.4 kg in BW. Animals were offered 11.4 kg/d per animal of concentrate diet containing equal amounts of soybean meal, whole cottonseed, and ground corn once a day with free access to hay (experiment 1) and 15.9 kg/d per animal of TMR consisting of silage, hay, corn, corn gluten, soybean meal, and minerals (experiment 2). Feed grab samples were collected, oven-dried, coarsely pulverized in a regular kitchen blender for one minute, and separated into three particle sizes (PS: 0.85, 1.4 and 2.36mm). The feed additives used were nitrate, fumarate and a nitrate-fumarate combination. Nitrate and ammonia concentrations were analyzed by FIA. The results of the study showed that the addition of NO_3 increased ($p < 0.05$) the pH, NO_3 and NH_3 concentrations. The addition of fumarate and the nitrate-fumarate combination had no effect on NO_3 and NH_3 concentrations. The addition of fumarate increased ($p < 0.05$) the pH in experiment 1, and fumarate had no effect on pH in experiment 2. The addition of the nitrate-fumarate combination increased the pH ($p < 0.05$) for both experiments. There was an increase ($p < 0.05$) in the pH for the medium PS in experiment 1, but there was no effect on pH by the small or large PS. There was also an increase ($p < 0.05$) in the pH for the medium and large PS in experiment 2, while the small PS had no effect on pH. The addition of nitrate and the nitrate-fumarate combination can

increase *in vitro* pH. The increase in the pH and ammonia concentrations by the addition of nitrate indicates that nitrate was reduced to ammonia.

Keywords: *in vitro, pH, ammonia, nitrate, fumarate*

4.1 Introduction

It has been indicated that the diet of ruminant animals can play a major role in altering the pH of the rumen. This change in rumen pH can influence methanogenesis and fermentation end products like VFAs (Van Kessel & Russell 1996; Lana et al., 1998; and Russell 1998). Previous research has focused on investigating the effects of pH on VFA and CH_4 production (Van Kessel & Russell 1996). Whereas it has also been reported that pH can have a major impact on the acetate to propionate ratios (Lana et al. 1998). Nitrate has also been studied as a feed additive in the ruminant diet (Sar et al., 2004; Zhou et al., 2011; Hulshof et al., 2012) and a report by Sar et al. (2004) indicated that the addition of NO_3 lowered ruminal pH and increased ruminal NH_3 concentrations in sheep. Also an increase in NH_3 concentrations by the addition of NO_3 was observed by Bozic et al. (2009); Zhou et al. (2011); and Hulshof et al. (2012). The addition of NO_3 as a feed additive on pH has had variable effects since studies have reported a decrease in ruminal pH Sar et al. (2004), while Zhou et al. (2011) reported an increase in pH as the dose of NO_3 increased. Fumarate is also another feed additive that has been studied as a feed additive that can alter the rumen environment and possibly have an impact on pH. Bayaru et al. (2000) and Yu et al. (2010) reported that fumarate had no effect on *in vivo* and *in vitro* pH, respectively. Also Wood et al. (2009) reported that encapsulated fumaric acid had no effect on pH. However, there are studies that indicate that the addition of fumarate can increase *in vitro* pH (Carro and Ranilla, 2003). Also the addition of fumarate has been shown to decrease NH_3 concentrations (Bayaru et al., 2000; Yu et al., 2010), but these results are variable since it has

been reported that fumarate has no effect on ammonia concentrations (Lopez et al., 1999; Mao et al., 2008). In addition to modifying the ruminant diet with feed additives, the manipulation of feed particle size can also have an impact on the rumen environment. The manipulation of feed can impact the rate of passage of feed and increase the surface area available for microorganisms to ferment, which has been shown to affect the pH of the rumen (Kononoff et al., 2003). Although the rate of passage of feed is related to the particle size of the feed it has been reported that the particle size of corn silage, chop length of forage, and particle size of grass hay had no effect on pH (Bhandari et al., 2007; Kononoff et al., 2003; Storm & Kristensen, 2010). However Teimouri et al. (2004) reported as forage particle size decreased the ruminal pH decreased and there was no effect on ruminal ammonia concentrations. The current study aimed to investigate if nitrate or fumarate and feed PS can influence the pH, NO_3 and NH_3 concentrations to determine if these feed additives can influence fermentation parameters that ultimately influence on methanogenesis. Therefore it is hypothesized that the addition of nitrate could increase *in vitro* pH, while fumarate decreases pH, and the nitrate-fumarate combination may have an intermediate effect on pH, while the addition of nitrate and the nitrate-fumarate combination will increase NH_3 concentrations. Also the feed particle size will influence *in vitro* pH.

4.2 Materials and Methods

4.2.1 Determination of Nitrate and Ammonia Production. Samples (20 mL) for ammonia and nitrate concentrations were collected 48h after incubation from each fermentation flask. The samples were centrifuged @ 4,000 rpm for 15 minutes at 4°C and frozen at -20°C until analysis. Samples were thawed and 8 ml aliquots were collected for each sample and filtered through Whatman® filter paper #41 (Sigma-Aldrich, St. Louis, MO, USA). The samples were analyzed for nitrate and ammonia using a colorimetric procedure on the Lachat Quickchem

800 Flow Injection Analyzer (FIA) (Loveland, CO, USA). All standards and reagents used were prepared using the QuickChem® Methods 12-107-06-2-A and 12-107-04-1-B for the determination of ammonia and nitrate using flow injection analysis. Nitrate standards were prepared at 200.0 mg N/L in 2M potassium chloride (0, 0.025, 0.50, 1.0, 2.0, 10.0, 20.0mL) and ammonia standards were prepared as 100.0 mg N/L in 2M potassium chloride (0, 0.5, 2.5, 10, 40, 100mL). The calibration curve was used to calculate sample concentration using the regression equation. If the samples exceeded above the calibration curve it was diluted and reanalyzed.

4.2.2 Determination of in vitro pH. The pH of *in vitro* fermentation fluids were measured routinely and the pH meter was standardized with buffer solutions with a pH of 10.00, 7.00, and 4.00(Fisher Scientific, USA). The pH was measured using the accumet Basic AB15 pH meter (Fisher Scientific, USA). The pH was measured immediately prior to leaving the farm and measured immediately upon returning to the university laboratory and then measured at 4, 24, and 48 hours post incubation to investigate the impact of nitrate and fumarate on pH during the 48 h incubation period.

4.2.3 Statistical Analysis and Experimental Design. The experiment was conducted in a randomized block design with animals as blocks, and three replications of the feed particle size with feed additive treatments per block. Data for the *in vitro* pH, NO_3, and NH_3 production were analyzed using SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). The data for pH, NO_3, and NH_3 were combined for each animal. Data for pH, NO_3, and NH_3 were analyzed with PROC MIXED of SAS, using a mixed model with animals as a random effect and particle size along with feed additive treatments as fixed effects (Little, Milliken, Stroup, Wolfinger, & Schabenberger, 2006; SAS Institute, 2012). The particle size by feed additive interaction was examined and where this interaction was not significant the effect of feed additives and particle

size were observed independently of each effect. All treatments were declared significant at ($p < 0.05$) and considered trends at ($p > 0.06$ and $p < 0.10$).

4.3 Results

4.3.1 Effect of Feed Particle Size and Feed Additives on pH. Table 13 shows the effect of feed particle size on *in vitro* fermentation fluid pH for experiments 1 and 2. The results show that the pH of the fluid was higher ($p < 0.05$) for the medium PS compared to the small PS and large PS in experiment 1. The small and the large PS were not significantly different each other. Also in experiment 2, *in vitro* pH increased ($p < 0.05$) for the medium and large PS compared to the small PS; however there was no significant difference between the medium and large PS. Although the pH increased for the medium PS in experiment 1 and the medium and large PS in experiment 2, there was no obvious trend observed for the effect of PS on *in vitro* pH in experiment 1, but the pH of fermentation fluid increased as the PS increased in experiment 2.

Table 13

Effects of Feed Particle Size on pH of the Fermentation Fluids

PS, mm	Small	Medium	Large	SEM
Experimental 1	6.78^{a}	7.06^{b}	6.78^{a}	0.1
Experimental 2	6.87^{a}	6.95^{b}	7.00^{b}	0.02

Note: Different superscripts within a row indicates means are different ($p<0.05$)

Table 14 shows the effects of feed additives on the pH of the fermentation fluid. In experiment 1, there was a pH increase ($p < 0.05$) by the addition of all feed additives, with nitrate having the highest increase ($p < 0.05$) followed by the nitrate-fumarate combination an then fumarate compared to the control, but there was no significant difference between fumarate and the nitrate-fumarate combination. Once again nitrate alone and in combination with fumarate increased ($p < 0.05$) the pH of the fermentation fluid compared to the control in experiment 2.

The addition of the nitrate-fumarate combination also increased (p <0.05) pH compared to fumarate, but there was no significant difference between the control and fumarate. In general the addition of nitrate and the nitrate-fumarate combination increased the pH, whereas the effect of fumarate on pH tended to be lower than the nitrate or nitrate-fumarate combination. The higher pH in the nitrate alone or in combination with fumarate did not come as a surprise because the reduction of nitrate to nitrite and then to ammonia will definitely raise the pH.

Table 14

Effects of Feed Additives on pH

	Control	Nitrate	Fumarate	Nitrate-Fumarate	SEM
Experiment 1	6.72^{a}	6.86^{b}	6.79^{c}	6.80^{c}	0.02
Experiment 2	6.66^{a}	6.89^{b}	6.65^{a}	6.78^{c}	0.03

Note: Different superscripts within a row indicates means are different (p<0.05)

4.3.2 Effect of Particle Size and Feed Additives on *in vitro* NO_3 and NH_3 Concentrations in Experiment 2.

Table 15 shows the effects of feed additives on in vitro NO_3 and NH_3 concentrations in experiment 2. The addition of nitrate increased ($p < 0.05$) NO_3 concentrations compared to the control, fumarate and the nitrate-fumarate combination. There was no effect on NO_3 concentrations by the addition of fumarate and the nitrate-fumarate combination compared to the control. Also there was no significant difference in NO_3 concentrations between fumarate and the nitrate-fumarate combination. The addition of nitrate increased ($p < 0.05$) NH_3 concentrations compared to the control, fumarate and the nitrate-fumarate combination. However, the addition of fumarate had no effect on NH_3 concentrations in the fumarate and the nitrate-fumarate combination which were also not significantly different from the control. The NH_3 concentrations for fumarate and the nitrate-fumarate combination

were not significantly different. Therefore the addition of nitrate increased the concentrations of NO_3 and NH_3, while fumarate and the nitrate-fumarate combination had no effect on NO_3 and NH_3 concentrations.

Table 15

Effect of Feed Additives on Nitrate and Ammonia Concentrations

Feed Additives	**Control**	**Nitrate**	**Fumarate**	**Nitrate-Fumarate**	**SEM**
Nitrate, ppm	8.95^{a}	224.4^{b}	9.04^{a}	9.18^{a}	53.8
Ammonia, ppm	1282.4^{a}	1703.1^{b}	1198.9^{a}	1182.3^{a}	122.5

Note: Different superscripts within a row indicates means are different ($p<0.05$)

Table 16

Effect of Particle Size on NH_3 Concentrations

Particle size, mm	**NO_3, ppm**	**SEM**	**NH_3, ppm**	**SEM**
0.85	119.5^{a}	33.8	1198.2^{a}	105.4
1.4	23.3^{b}	33.8	1361.3^{b}	105.4
2.36	13.5^{b}	33.8	996.8^{c}	105.4

Note. Different superscripts in columns indicate means that are different ($p < 0.05$)

Table 16 shows the effect of TMR PS on NO_3 and NH_3 concentrations. Nitrate concentrations were higher ($p < 0.05$) for the small PS compared to the medium and large PS, but there was no significant difference between the later. Although the NO_3 concentrations for the medium PS were 42% higher than the large PS there was no statistical difference between the medium and the large PS. Ammonia concentrations for the three PS was in the order of medium, small , large and differences were significant at ($p < 0.05$). It is less clear why the medium PS produced more NH_3 than the small particle size, but relative to the large particle size it might

mean there might have been more surface area for microbial attachment than the larger particle size

4.4 Discussion

This study investigated the effects of feed additives and feed particle sizes on changes *in vitro* for pH, NO_3, and NH_3 concentrations. In the current study the addition of nitrate increased the *in vitro* pH for both experiments. Our findings in the current study are in agreement with Zhou et al. (2011) who reported an increase in pH with increases in nitrate. In experiment 1, the addition of fumarate increased the pH and this is also in agreement with Carro and Ranilla, (2003) who reported that the addition of fumarate increased *in vitro* pH. However, the results in experiment 2 contradict with the results from experiment 1 since the addition of fumarate had no effect on the pH. This finding is supported by Yu et al. (2010) who also reported that fumarate had no effect on *in vitro* pH. The increase in pH by the addition of fumarate in experiment 1 in the current study is not clearly understood. However, an increase in VFA concentrations in the rumen has been associated with a reduction in ruminal pH (Dijkstra et al. 2012). If this is taken into account then in experiment 2 the lack of influence of fumarate on the pH could be related to fumarate not having an impact on VFA production.

The increase in pH by the addition of nitrate in our study could be possibly explained by the decrease in VFA production and most likely by the increase in ammonia production (Zhou et al., 2011). The addition of the nitrate-fumarate combination had no effect on NO_3 and NH_3 concentrations. Iwamoto et al. (1999) reported that the addition of fumarate to nitrate increased the rate of NO_3 reduction, and Hulshof et al. (2012) also indicated that NO_3 is reduced to NH_3. Therefore, an increase in NH_3 concentrations by the addition of the nitrate-fumarate combination was expected to be observed in the current study. However, Bayaru et al. (2000) reported that

fumarate reduced NH_3 concentrations suggesting that fumarate increased the utilization of nitrogen for bacteria by providing energy for the rumen microorganisms to improve utilization of ammonia. Also Yu et al., (2010) reported that fiber degrading bacteria are known to be enhanced by the addition of fumarate. These reports may explain why there was no difference in NO_3 and NH_3 concentrations observed by the addition of fumarate or the nitrate-fumarate combination. However, our study did not measure nitrite reduction, but the lack of effect of the nitrate-fumarate combination on NO_3 and NH_3 concentrations does not support the idea that NO_3 was rapidly reduced to NH_3.

In experiment 1 the medium PS increased the pH. In experiment 2 there was a similar increase in pH for the medium but also for the large PS. An increase in VFA production can lower the pH (Dijkstra et al., 2012) and if the increased VFA production observed in the small PS in experiment 2 of our study is taken into account then that may explain the lower pH readings recorded for the small PS. The results of the current study also revealed that the small PS had the highest amounts of NO_3, while the medium and large PS had lower NO_3 concentrations. There was also no effect of PS on NH_3 concentrations. This finding is in agreement with (Kononoff & Heinrichs, 2003; Teimouri et al., 2004) who also observed no effect of alfalfa PS on NH_3 concentrations in Holstein cows.

4.5 Conclusion

Nitrate and the combination of nitrate and fumarate consistently increased *in vitro* pH indicating that nitrate alone or in combination with fumarate played a role in changing the rumen environment. The increase in the ammonia concentrations by the addition of nitrate indicates that nitrate-reducing bacteria were able to reduce nitrate to ammonia. Consequently the increase in pH is related to an increase in ammonia production. The absence of the accumulation of

ammonia by the addition of the nitrate-fumarate combination suggests that fumarate may have increased the utilization of ammonia by fiber-degrading bacteria as a source of energy for their growth. Therefore, adding fumarate to nitrate can be a benefit by reducing the accumulation of ammonia. Also reducing the feed particle size reduced the pH which supports an increase in VFA production observed in our study. In conclusion the addition of fumarate with nitrate can be a beneficial feed additive to promote increased utilization of ammonia by fiber degrading bacteria and this supports the idea that the suppression of methanogenesis is due to the nitrate reducing bacteria out-competing methanogens for free hydrogen for methane synthesis.

CHAPTER 5

Summary and Conclusion

5.1 Summary and Discussion

The objective was to determine the effects of feed particle size and nitrate or fumarate alone or in combination on methane production. The current study also aimed to determine the effects of feed particle size and adding nitrate or fumarate to the diet on *in vitro* VFA production and other fermentation end products (pH, VFA, NO_3, and NH_3) as well as methanogen abundances. It was hypothesized that the addition of nitrate and fumarate alone or in combination reduces *in vitro* methane production and the nitrate-fumarate provides a greater reduction in methane production by simply reducing the availability of free H_2 for methanogens and reduce their abundances. It was also hypothesized that a nitrate-fumarate combination would have the capacity to increase the production of propionate without adversely affecting VFA concentrations and also that feed particle size would influence *in vitro* fermentation parameters and CH_4 production in particular. The research conducted in this study has addressed these hypotheses.

It has been recognized that ruminant animals contribute significantly to atmospheric methane production. Rececently, feed additves such as nitrate and fumarate have been tested for their ability to reduce enteric methane production (Beauchemin et al., 2006 and Hulshof et al., 2002). However, high doses of nitrate in the ruminant diet raises the risk of methemoglobinemia due to the accumulation of nitrite by rapid degradation of nitrate (van Zijderveld et al., 2010). This risk in nitrite toxicity has led to the investigation of the combination of nitrate with other feed additives like sulfate (van Zijderveld et al., 2010), prebiotics (Sar et al., 2004), and fumarate

(Iwamoto et al., 1999) to counteract this undersirable effect from the reduction of nitrate in ruminants.

In the current study we found that the addition of nitrate consistently and effectivley reduced *in vitro* methane production by 58%. This finding is in agreement with (Bozic et al., 2009 and Zhou et al., 2011) who reported nitrate decreased *in vitro* methane production. Also we found that the addition of nitrate reduced individual and total VFA production. It was also noted that the addition of nitrate increased the acetate to propionate ratios in both experiments. It was suggested that the intermediate nitrite can suppress microbial fermentation therefore explaining the decrease in methane production (Iwamoto et al., 1999). Overall the administration of nitrate resulted in an increase in pH and ammonia concentrations. Therefore, the increase in pH may be caused by the increase in ammonia production from respiratory nitrate ammonification (Zhou et al., 2011).

The current study also showed that the addition of fumarate to the did not affect *in vitro* methane or total VFA production. This observation that fumarate had no effect on methane production was also reported by Beauchemin and McGinn (2006a and 2006b). Although the addition of fumarate increased the production of propionate in experiment 2, the absence of an effect on total VFA production did not agree with Beachemin and McGinn (2006a and 2006b) who reported that the addition of fumaric acid increased propionate and total VFA production. The inconsistencies in the results from the current study and the study by Beauchemin (2006a and 2006b) could be due to the fact that *in vitro* measurements indicate VFA production whereas *in vivo* measurements indicates the balance between VFA production and absorption in ruminant animals. Also fumarate had no effect on the acetate to propionate ratios in both experiments. The fact that fumarate had no effect on the pH or ammonia concentrations in the current study

indicates that fumarate may have increased the utilization of ammonia by fiber-degrading rumen bacteria (Yu et al., 2010).

Likewise, the addition of the nitrate-fumarate combination consistenly decreased methane production by as much as 40% and 68% in experiments 1 and 2, respectively. In experiment1, the addition of the nitrate-fumarate combination had no effect on the production of acetate, propionate, valerate and iso-valerate. Likewise the addition of the nitrate-fumarate combination had no effect on total volatile fatty acid production in experiment 1 and on propionate and iso-butyrate production in experiment 2. However, the addition of the nitrate-fumarate combination decreased the production of acetate, butyrate, valerate and iso-valerate in experiment 2 with no significant effect on the acetate to propionate ratios in both experiments. The addition of the nitrate-fumarate combination also increased *in vitro* pH. Although pH can be an indicator of ammonia production (Yu et al., 2010) there was no increase in the production of ammonia by the addition of the nitrate-fumarate combination. It was reported by Yu et al. (2010) that the addition of fumarate can increase the utilization of ammonia by ruminal microorganisms and could possibly explain why we did not see an increase in ammonia concentrations although we observed an increase in the pH.

Reducing the feed particle size had variable effects on methane production. For instance, in experiment 1 methane production for the medium PS was lower ($p<0.05$) than the small and large particle size. Whereas in experiment 2, methane production was higher ($p<0.05$) for the large particle size compared to the small and medium particle sizes. Therefore, although the effect of CH_4 production was evident, there was no definite pattern that could be associated with any specific particle size used in the study. Also reducing the feed particle size had variable effects on volatile fatty acid production. However in experiment 2, production of the three major

volatile fatty acids (acetate, propionate, and butyrate) increased as the particle size decreased. There was also an increase in the pH by the medium particle size in experiment 1 and by the medium and large particle size in experiment 2. Therefore as the feed particle size decreased the *in vitro* pH decreased and coulde be an indication of increased VFA production. There was no definite trend observed for the effect of feed particle size on ammonia concentrations.

The addition of the feed additives individually or in combination had no significant effect on total methanogens. This finding agrees with Mohammed et al. (2011) who reported that total methanogens did not differ among diets. However, the addition of the feed additives increased the relative abundance of *Methanobrevibacter sp.* AbM4 and M. *stadtmanae*. The addition of fumarate decreased ($p<0.05$) the relative abundance of *Methanobrevibacter sp.* AbM4 compared to the nitrate or nitrate-fumarate combination, while nitrate decreased ($p<0.05$) the abundance of M. *stadtmanae* compared to fumarate. The effect of the addition of fumarate on M. AbM4 in the current study suggests that the addition of fumarate may have stimulated fumarate reducing bacteria that competed with these species for the substrates hydrogen and formate for methanogenesis (Yang et al., 2012). Also the effect of the addition of nitrate on M. *stadtmanae* suggests that this species could be sensitive to nitrate since it depends on hydrogen and methanol as substrates for methanogenesis (Fricke et al., 2006). The fact that the total methanogens in the study were not affected by the addition of the feed additives while the abundances of the two methanogen species increased in our study may suggest that these two species may not contribute significantly to the total methanogen population present in the study.

In summary, the results of the study indicate that nitrate alone or in combination with fumarate can effectively reduced CH_4 production. However, the decrease in the production of VFAs by the addition of nitrate may suggest that this feed additive be used in combination with

another feed additive like fumarate to alleviate the decrease in VFA production. The increase in pH by the addition of nitrate and the nitrate-fumarate combination indicates that ammonia production occurred and the high concentrations of ammonia may suggest that nitrate reducing bacteria reduced nitrate to ammonia and this reduction of nitrate and its intermediate nitrite may have directly or indirectly reduced methane production. The absence of the accumulation of ammonia by the addition of fumarate and the nitrate-fumarate combination suggests that fumarate may have increased the utilization of ammonia by fiber-degrading bacteria. Therefore, the use of the nitrate-fumarate combination needs to be further investigated to better understand the role that fumarate may have played in influencing nitrate and ammonia concentrations.

5.2 Conclusion

The present study demonstrated that the addition of nitrate alone or in combination with fumarate effectively reduced CH_4 production, with nitrate being mainly responsible for effectively reducing *in vitro* methane production. In addition fumarate could be useful when combined with nitrate to prevent a decrease in VFA production, but the mode of action for the combination of nitrate with fumarate is not yet known. Overall the combination of fumarate with nitrate has the potential to reduce methanogenesis without the possibility of suppressing *in vitro* microbial fermentation.

5.3 Recommendations for Future Work

Variations in CH_4 production differs between animals even when the diet remains fairly constant. Although the animals in the study were treated as representatives of the dairy herd at the farm, the animal-to-animal variations could be reduced by increasing the number of animals in the study. Real-time PCR can also be performed with more replications to further understand how the methanogen species change by treatment. Further work is required to understand the

variations in using the nitrate-fumarate combination as a suitable feed additive to reduce CH_4 emissions. Although authors have indicated that fumarate can enhance nitrate and nitrite accumulation we did not investigate the effect of fumarate on nitrite accumulation. This area deserves more attention and could be benefitial as the fear of nitrite toxicity in ruminants is still a major concern when introducing high nitrate containing feeds to ruminant animals. The rumen microbial communities are diverse and there are still methanogens species that have not been fully identified. Therefore, focusing on other methanogen species like *Methanobrevibacter ruminantium, Methanobacterium formicicum, Methanosarcina barkeri,* and *Methanomicrobium mobile* can give further insight into how the diversity of the methanogen population may change in the presence of the feed additives used in the study. Future studies should investigate the effects of incorporating the combination of nitrate and fumarate in the diets of ruminant animals on the general rumen microbial populations including nitrate and fumarate reducing bacteria and the methanogen communities in particular.

References

Abdl-Rahman, M. A., Sawiress, F. A. R., & Abd El-Aty, A. M. (2010). Effect of sodium lauryl sulfate-fumaric acid coupled addition on the in vitro rumen fermentation with special regard to methanogenesis. *Veterinary Medicine International, 2010*. doi: 10.4061/2010/858474

Alemu, A.W., Dijkstra, J., Bannink, A., France, J., Kebreab, E. (2011). Rumen stoichiometric models and their contribution and challenges in predicting enteric methane production. *Animal Feed Science and Technology,*166-167, 761-778.

Anderson, R. C., & Rasmussen, M. A. (1998). Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. *Bioresource Technology, 64*(2), 89-95. doi: 10.1016/s0960-8524(97)00184-3

Anderson, R. C., Huwe, J. K., Smith, D. J., Stanton, T. B., Krueger, N. A., Callaway, T. R., . . . Nisbet, D. J. (2010). Effect of nitroethane, dimethyl-2-nitroglutarate and 2-nitro-methyl-propionate on ruminal methane production and hydrogen balance in vitro. *Bioresource Technology, 101*(14), 5345-5349. doi: 10.1016/j.biortech.2009.11.108

Araújo, W. L., Nunes-Nesi, Adriano, & Fernie, Alisdair R. (2011). Fumarate: Multiple functions of a simple metabolite. *Phytochemistry, 72*(9), 838-843. doi: 10.1016/j.phytochem.2011.02.028

Asanuma, N., Iwamoto, M., & Hino, T. (1999). Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. *Journal of Dairy Science, 82*(4), 780-787. doi: 10.3168/jds.S0022-0302(99)75296-3

Attwood, G. T., Altermann, E., Kelly, W. J., Leahy, S. C., Zhang, L., & Morrison, M. (2011). Exploring rumen methanogen genomes to identify targets for methane mitigation

strategies. *Animal Feed Science and Technology, 166-167*(0), 65-75. doi: 10.1016/j.anifeedsci.2011.04.004

Bayaru, E., Kanda, S., Kamada, T., Itabashi, H., Andoh, S., Nishida, T., . . . Isobe, Y. (2000). Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. *Animal Science Journal, 72*(2), 139-146.

Beauchemin, K. A., & McGinn, S. M. (2006a). Effects of various feed additives on the methane emissions from beef cattle. *International Congress Series, 1293*(0), 152-155. doi: http://dx.doi.org/10.1016/j.ics.2006.01.042

Beauchemin, K. A., & McGinn, S. M. (2006b). Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. *Journal of Animal Science, 84*(6), 1489-1496.

Bell, M. J., Wall, E., Russell, G., Simm, G., & Stott, A. W. (2011). The effect of improving cow productivity, fertility, and longevity on the global warming potential of dairy systems. *Journal of Dairy Science, 94*(7), 3662-3678. doi: 10.3168/jds.2010-4023

Bhandari, S. K., Li, S., Ominski, K. H., Wittenberg, K. M., & Plaizier, J. C. (2008). Effects of the chop lengths of alfalfa silage and oat silage on feed intake, milk production, feeding behavior, and rumen fermentation of dairy cows. *Journal of Dairy Science, 91*(5), 1942-1958. doi: 10.3168/jds.2007-0358

Blümmel, M., Steingrass, H., Becker, K, Soller, H. (1993). Production of SCFA, CO_2, CH_4 and microbial cells in vitro, *Proceedings of the Society of Nutrition Physiology*, 1-9

Blümmel, M., Aiple, K. P., Steingrass, H., & Becker, K. (1999). A note on the stoichiometrical relationship of short chain fatty acid production and gas formation in vitro in feedstuffs of widely differing quality. *Journal of Animal Physiology and Animal Nutrition, 81*(3), 157-167. doi: 10.1046/j.1439-0396.1999.813205.x

Blümmel, M., Givens, D. I., Moss,A. R. (2005). Comparison of methane produced by straw fed sheep in open-circuit respiration with methane predicted by fermentation characteristics measured by an in vitro gas procedure. *Animal Feed Science and Technology,* 123-124, 379-390.doi:10.1016/j.anifeedsci.2005.06.001

Boadi, D.A, & Wittenberg, K. M. (2002). Methane production from dairy and beef heifers fed forages differing in nutrient density using the sulphur hexafluoride (SF6) tracer gas technique. *Canadian Journal of Animal Science, 82(2),* 201-206. Doi: 10.4141/A01-017

Boadi, D., Benchaar, C., Chiquette, J., & Masse, D. (2004b). Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. *Canadian Journal of Animal Science, 84*(3), 319-335.

Božic, A. K., Anderson, R. C., Carstens, G. E., Ricke, S. C., Callaway, T. R., Yokoyama, M. T., . . . Nisbet, D. J. (2009). Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin® and the Hawaiian marine algae Chaetoceros on ruminal fermentation in vitro. *Bioresource Technology, 100*(17), 4017-4025. doi: 10.1016/j.biortech.2008.12.061

Carro, M. D., & Ranilla, M. J. (2003). Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms *in vitro*. *British Journal of Nutrition, 90*, 617-623. doi: 10.1079/BJN2003935

Castillo, C., Benedito, J. L., Méndez, J., Pereira, V., López-Alonso, M., Miranda, M., & Hernández, J. (2004). Organic acids as a substitute for monensin in diets for beef cattle. *Animal Feed Science and Technology, 115*(1–2), 101-116. doi: 10.1016/j.anifeedsci.2004.02.001

Castro-Montoya, J., De Campeneere, S., Van Ranst, G., & Fievez, V. (2012). Interactions between methane mitigation additives and basal substrates on in vitro methane and VFA

production. *Animal Feed Science and Technology, 176*(1–4), 47-60. doi: http://dx.doi.org/10.1016/j.anifeedsci.2012.07.007

Costa, K. C., Lie, Thomas J., Jacobs, M. A., & Leigh, J. A. (2013). H2-Independent growth of the hydrogenotrophic methanogen methanococcus maripaludis. *mBio, 4*(2). doi: 10.1128/mBio.00062-13

Dijkstra, J., Ellis, J. L., Kebreab, E., Strathe, A. B., López, S., France, J., & Bannink, A. (2012). Ruminal pH regulation and nutritional consequences of low pH. *Animal Feed Science and Technology, 172*(1–2), 22-33. doi: 10.1016/j.anifeedsci.2011.12.005

Dijkstra, J., Boer, H., Van Bruchem, J., Bruining, M., & Tamminga, S. (1993). Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *British Journal of Nutrition, 69*(02), 385-396. doi: doi:10.1079/BJN19930041

EPA, U.S. (2012). *Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2010.* Washington, DC.

EPA. (2012). Sources of Greenhoue Gas Emissions. Retrieved June 14, 2012, from http://www.epa.gov/climatechange/ghgemissions/sources.html

Farra, P. A., & Satter, L. D. (1971). Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. *Journal of Dairy Science, 54*(7), 1018-1024.

Foley, P. A., Kenny, D. A., Lovett, D. K., Callan, J. J., Boland, T. M., & O'Mara, F. P. (2009). Effect of dl-malic acid supplementation on feed intake, methane emissions, and performance of lactating dairy cows at pasture. *Journal of Dairy Science, 92*(7), 3258-3264. doi: 10.3168/jds.2008-1633

Fricke, W. F., Seedorf, H., Henne, A., Kruer, M., Liesegang, H., Hedderich, R., . . . Thauer, R. K. (2006). The genome sequence of Methanosphaera stadtmanae reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. *J Bacteriol, 188*(2), 642-658. doi: 10.1128/jb.188.2.642-658.2006

Grainger, C., & Beauchemin, K. A. (2011). Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal Feed Science and Technology, 166–167*(0), 308-320. doi: 10.1016/j.anifeedsci.2011.04.021

Grainger, C., Williams, R., Clarke, T., Wright, A. D. G., & Eckard, R. J. (2010). Supplementation with whole cottonseed causes long-term reduction of methane emissions from lactating dairy cows offered a forage and cereal grain diet. *Journal of Dairy Science, 93*(6), 2612-2619. doi: 10.3168/jds.2009-2888

Grainger, C., Williams, R., Eckard, R. J., & Hannah, M. C. (2010). A high dose of monensin does not reduce methane emissions of dairy cows offered pasture supplemented with grain. *Journal of Dairy Science, 93*(11), 5300-5308. doi: 10.3168/jds.2010-3154

Henderson, Gemma, Cox, Faith, Kittelmann, Sandra, Miri, Vahideh Heidarian, Zethof, Michael, Noel, Samantha J., . . . Janssen, Peter H. (2013). Effect of DNA Extraction Methods and Sampling Techniques on the Apparent Structure of Cow and Sheep Rumen Microbial Communities. *PLoS ONE, 8*(9), e74787. doi: 10.1371/journal.pone.0074787

Hernandez-Sanabria, Emma, Guan, Le Luo, Goonewardene, Laksiri A., Li, Meiju, Mujibi, Denis F., Stothard, Paul, . . . Leon-Quintero, Monica C. (2010). Correlation of particular bacterial pcr-denaturing gradient gel electrophoresis patterns with bovine ruminal fermentation parameters and feed efficiency traits. *Applied and Environmental Microbiology, 76*(19), 6338-6350. doi: 10.1128/aem.01052-10

Hook, S. E., Wright, A. D. G., & McBride, B. W. (2010). Methanogens: Methane producers of the rumen and mitigation strategies. *Archaea-an International Microbiological Journal*. doi: 10.1155/2010/945785

Hook, S., Steele, M., Northwood, K., Wright, A.-D., & McBride, B. (2011). Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. *Microbial Ecology, 62*(1), 94-105. doi: 10.1007/s00248-011-9881-0

Hulshof, R. B. A., Berndt, A., Gerrits, W. J. J., Dijkstra, J., van Zijderveld, S. M., Newbold, J.R., & Perdok, H. B. (2012). Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane based diets. *Journal of Animal Science*. doi: 10.2527/jas.2011-4209

IPCC. (2007). Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, 2007. In S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor & H. L. Miller (Eds.). Cambridge, United Kingdom and New York, NY, USA.

Iqbal, M. F., Cheng, Y. F., Zhu, W. Y., & Zeshan, B. (2008). Mitigation of ruminant methane production: Current strategies, constraints and future options. *World Journal of Microbiology and Biotechnology, 24*(12), 2747-2755.

Iwamoto, M., Asanuma, N., & Hino, T. (1999). Effects of nitrate combined with fumarate on methanogenesis, fermentation, and cellulose digestion by mixed ruminal microbes *in vitro*. *Animal Science Journal, 70*(6), 471-478.

Iwamoto, M., Asanuma, N., & Hino, T. (2002). Ability of Selenomonas ruminantium, Veillonella parvula, and Wolinella succinogenes to reduce nitrate and nitrite with special

reference to the suppression of ruminal methanogenesis. *Anaerobe, 8*(4), 209-215. doi: 10.1006/anae.2002.0428

Janssen, P. H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology, 160*(1-2), 1-22. doi: 10.1016/j.anifeedsci.2010.07.002

Janssen, P. H., & Kirs, M. (2008). Structure of the archaeal community of the rumen. *Applied and Environmental Microbiology, 74*(12), 3619-3625. doi: 10.1128/aem.02812-07

Jayanegara, A., Togtokhbayar, N., Makkar, H. P. S., & Becker, K. (2009). Tannins determined by various methods as predictors of methane production reduction potential of plants by an in vitro rumen fermentation system. *Animal Feed Science and Technology, 150*(3–4), 230-237. doi: 10.1016/j.anifeedsci.2008.10.011

Johnson, Ronald R. (1966). Techniques and Procedures for In Vitro and In Vivo Rumen Studies. *Journal of Animal Science, 25*(3), 855-875.

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of Animal Science, 73*(8), 2483–2492.

Klüber, H. D., & Conrad, R. (1998). Inhibitory effects of nitrate, nitrite, NO and N_2O on methanogenesis by Methanosarcina barkeri and Methanobacterium bryantii. *FEMS Microbiology Ecology, 25*(4), 331-339. doi: 10.1016/S0168-6496(97)00102-5

Knight, T., Ronimus, R. S., Dey, D., Tootill, C., Naylor, G., Evans, P., . . . Clark, H. (2011). Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. *Animal Feed Science and Technology, 166-167*(0), 101-112. doi: 10.1016/j.anifeedsci.2011.04.059

Kononoff, P. J., & Heinrichs, A. J. (2003a). The Effect of Corn Silage Particle Size and Cottonseed Hulls on Cows in Early Lactation. *Journal of dairy science, 86*(7), 2438-2451.

Kononoff, P. J., & Heinrichs, A. J. (2003b). The Effect of Reducing Alfalfa Haylage Particle Size on Cows in Early Lactation. *Journal of dairy science, 86*(4), 1445-1457.

Kononoff, P. J., Heinrichs, A. J., & Lehman, H. A. (2003). The Effect of Corn Silage Particle Size on Eating Behavior, Chewing Activities, and Rumen Fermentation in Lactating Dairy Cows. *Journal of Dairy Science, 86*(10), 3343-3353. doi: http://dx.doi.org/10.3168/jds.S0022-0302(03)73937-X

Krause, K. M., & Combs, D. K. (2003). Effects of Forage Particle Size, Forage Source, and Grain Fermentability on Performance and Ruminal pH in Midlactation Cows. *Journal of dairy science, 86*(4), 1382-1397.

Kumar, Sanjay, Dagar, SumitSingh, & Puniya, AnilKumar. (2012). Isolation and characterization of methanogens from rumen of Murrah buffalo. *Annals of Microbiology, 62*(1), 345-350. doi: 10.1007/s13213-011-0268-8

Lana, R. P., Russell, J. B., & Van Amburgh, M. E. (1998). The role of pH in regulating ruminal methane and ammonia production. *Journal of Animal Science, 76*(8), 2190-2196.

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., . . . Attwood, G. T. (2010). The Genome sequence of the rumen methanogen Methanobrevibacter ruminantium reveals new possibilities for controlling ruminant methane emissions. *Plos One, 5*(1). doi: 10.1371/journal.pone.0008926

Leahy, Sinead Christine, Kelly, William J, Li, Dong, Li, Yang, Altermann, Eric, Lambie, SC, . . . Attwood, GT. (2013). The complete genome sequence of Methanobrevibacter sp. AbM4. *Standards in genomic sciences, 8*(2), 215.

Leng, R. A., & Nolan, J. V. (1984). Nitrogen metabolism in the rumen. *Journal of Dairy Science, 67*(5), 1072-1089. doi: 10.3168/jds.S0022-0302(84)81409-5

Li, M., Gong, J., Cottrill, M., Yu, H., de Lange, C., Burton, J., & Topp, E.. (2003). Evaluation of QIAamp® DNA Stool Mini Kit for ecological studies of gut microbiota. *Journal of Microbiological Methods, 54*(1), 13-20. doi: 10.1016/S0167-7012(02)00260-9

Littell, R. C., Milliken, G. A., Wolfinger, R. D., & Schabenberger, O. (2006). *SAS for mixed models* (2nd ed.).

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. *Methods, 25*(4), 402-408. doi: 10.1006/meth.2001.1262

Lopez, S., Valdes, C. , Newbold, C. J., & Wallace, R. J. (1999). Influence of sodium fumarate addition on rumen fermentation *in vitro*. *British Journal of Nutrition, 81*, 59-64.

Machmüller, A. (2006). Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. *Agriculture, Ecosystems & Environment, 112*(2–3), 107-114. doi: 10.1016/j.agee.2005.08.010

Mao, S. Y., Zhang, G., & Zhu, W. Y. (2008). Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *Animal Feed Science and Technology, 140*(3–4), 293-306. doi: 10.1016/j.anifeedsci.2007.04.001

McAllister, T. A., Cheng, K. J., Okine, E. K., & Mathison, G. W. (1996). Dietary, environmental and microbiological aspects of methane production in ruminants. *Canadian Journal of Animal Science, 76*(2), 231-243. doi: 10.4141/cjas96-035

McCartney, C. A., Bull, I. D., Waters, S. M., & Dewhurst, R. J. (2013). Technical note: Comparison of biomarker and molecular biological methods for estimating methanogen abundance. *Journal of Animal Science, 91*(12), 5724-5728. doi: 10.2527/jas.2013-6513

McSweeney, C. S., Denman, S. E., Wright, A. D. G., & Yu, Z. (2007). Application of recent DNA/RNA-based techniques in rumen ecology. *Asian-Australasian Journal of Animal Sciences, 20*(2), 283-294.

Mohammed, R., Zhou, M., Koenig, K. M., Beauchemin, K. A., & Guan, L. L. (2011). Evaluation of rumen methanogen diversity in cattle fed diets containing dry corn distillers grains and condensed tannins using PCR-DGGE and qRT-PCR analyses. *Animal Feed Science and Technology, 166-167*(0), 122-131. doi: 10.1016/j.anifeedsci.2011.04.061

Morgavi, D. P., Forano, E., Martin, C., & Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal, 4*(7), 1024-1036. doi: 10.1017/s1751731110000546

Morvay, Y., Bannink, A., France, J., Kebreab, E., & Dijkstra, J. (2011). Evaluation of models to predict the stoichiometry of volatile fatty acid profiles in rumen fluid of lactating Holstein cows. *Journal of Dairy Science, 94*(6), 3063-3080. doi: 10.3168/jds.2010-3995

Moss, A. R, Jouany, J.-P., & Newbold, J. (2000). *Methane production by ruminants: Its contribution to global warming.* Paper presented at the Annales de Zootechnie.

Münger, A., & Kreuzer, M. (2006). Methane emission as determined in contrasting dairy cattle breeds over the reproduction cycle. *International Congress Series, 1293*(0), 119-122. doi: http://dx.doi.org/10.1016/j.ics.2006.01.072

Mwenya, B., Sar, C., Pen, B., Morikawa, R., Takaura, K., Kogawa, S., . . . Takahashi, J. (2006). Effect of feed additives on ruminal methanogenesis and anaerobic fermentation of manure in cows and steers. *International Congress Series, 1293*(0), 209-212. doi: 10.1016/j.ics.2006.03.027

Oppermann, R. A., Nelson, W. O., & Brown, R. E. (1957). *In vitro* studies on methanogenic rumen bacteria. *Journal of Dairy Science, 40*(7), 779-788. doi: 10.3168/jds.S0022-0302(57)94554-X

Ozutsumi, Y., Tajima, K., Takenaka, A., & Itabashi, H. (2006). Real-time PCR detection of the effects of protozoa on rumen bacteria in cattle. *Current Microbiology, 52*(2), 158-162. doi: 10.1007/s00284-005-0266-9

Patra, A. K., & Saxena, J. (2010). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry, 71*(11-12), 1198-1222. doi: 10.1016/j.phytochem.2010.05.010

Pinares-Patiño, C. S., Waghorn, G. C., Machmüller, A., Vlaming, B., Molano, G., Cavanagh, A., & Clark, H. (2007). Methane emissions and digestive physiology of non-lactating dairy cows fed pasture forage. *Canadian Journal of Animal Science, 87*(4), 601-613. doi: 10.4141/CJAS06023

Pinares-Patiño, C. S., Machmüller, A., Molano, G., Smith, A., Vlaming, J. B., & Clark, H. (2008). The SF6 tracer technique for measurements of methane emission from cattle –

effect of tracer permeation rate. *Canadian Journal of Animal Science, 88*(2), 309-320. doi: 10.4141/CJAS07117

Russell, J. B. (1998). The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production *in vitro*. *Journal of Dairy Science, 81*(12), 3222-3230. doi: 10.3168/jds.S0022-0302(98)75886-2

Sar, C., Santoso, B., Mwenya, B., Gamo, Y., Kobayashi, T., Morikawa, R., . . . Takahashi, J. (2004). Manipulation of rumen methanogenesis by the combination of nitrate with β1-4 galacto-oligosaccharides or nisin in sheep. *Animal Feed Science and Technology, 115*(1–2), 129-142. doi: 10.1016/j.anifeedsci.2004.01.006

SAS Institute. SAS/STAT® 12.1 user's guide. North Carolina: SAS Institute; 2012.

Sharp, R., Ziemer, C. J., Stern, M. D., & Stahl, D. A. (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiology Ecology, 26*(1), 71-78. doi: 10.1016/s0168-6496(98)00024-5

Shibata, Masaki, & Terada, Fuminori. (2010). Factors affecting methane production and mitigation in ruminants. *Animal Science Journal, 81*(1), 2-10. doi: 10.1111/j.1740-0929.2009.00687.x

Shin, E. C., Choi, B. R., Lim, W. J., Hong, S. Y., An, C. L., Cho, K. M., . . . Yun, H. D. (2004). Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe, 10*(6), 313-319. doi: 10.1016/j.anaerobe.2004.08.002

Siciliano-Jones, J., & Murphy, M. R. (1989). Production of volatile fatty acids in the rumen and cecum-colon of steers as affected by forage: Concentrate and forage physical form. *Journal of Dairy Science, 72*(2), 485-492. doi: 10.3168/jds.S0022-0302(89)79130-X

Skillman, L. C., Evans, P. N., Naylor, G. E., Morvan, B., Jarvis, G. N., & Joblin, K. N. (2004). 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe, 10*(5), 277-285. doi: 10.1016/j.anaerobe.2004.05.003

Spanghero, M., Zanfi, C., Fabbro, E., Scicutella, N., & Camellini, C. (2008). Effects of a blend of essential oils on some end products of in vitro rumen fermentation. *Animal Feed Science and Technology, 145*(1–4), 364-374. doi: 10.1016/j.anifeedsci.2007.05.048

Storm, A. C., & Kristensen, N. B. (2010). Effects of particle size and dry matter content of a total mixed ration on intraruminal equilibration and net portal flux of volatile fatty acids in lactating dairy cows. *Journal of Dairy Science, 93*(9), 4223-4238. doi: 10.3168/jds.2009-3002

Sutton, J. D., Dhanoa, M. S., Morant, S. V., France, J., Napper, D. J., & Schuller, E. (2003). Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. *Journal of Dairy Science, 86*(11), 3620-3633. doi: http://dx.doi.org/10.3168/jds.S0022-0302(03)73968-X

Teimouri Yansari, A., Valizadeh, R., Naserian, A., Christensen, D. A., Yu, P., & Eftekhari Shahroodi, F. (2004). Effects of alfalfa particle size and specific gravity on chewing activity, digestibility, and performance of holstein dairy cows. *Journal of Dairy Science, 87*(11), 3912-3924.

Tilley, J. M. A., & Terry, R. A. (1963). A two-stage technique for the in vitro digestion of forage crops. *Grass and Forage Science, 18*(2), 104-111. doi: 10.1111/j.1365-2494.1963.tb00335.x

Tokura, M., Ushida, K., Miyazaki, K., & Kojima, Y. (1997). Methanogens associated with rumen ciliates. *FEMS Microbiology Ecology, 22*(2), 137-143. doi: 10.1016/s0168-6496(96)00084-0

UNFCCC. (2012). *Background on the UNFCCC: The international response to climate change.* Retrieved June 2, 2012, from http://www.unfccc.int/essential_background/items/6031.php

Ungerfeld, E. M., Kohn, R. A., Wallace, R. J., & Newbold, C. J. (2007). A meta-analysis of fumarate effects on methane production in ruminal batch cultures. *Journal of Animal Science, 85*(10), 2556-2563. doi: 10.2527/jas.2006-674

Van Kessel, J. A. S., & Russell, J. B. (1996). The effect of pH on ruminal methanogenesis. *FEMS Microbiology Ecology, 20*(4), 205-210. doi: 10.1016/0168-6496(96)00030-x

Van Soest, P. J. (1963). Use of detergents in the analysis of fibrous feeds. A rapid method for the determination of fiber and lignin. *Journal of the Association of Official Agricultural Chemists, 46*, 829-835.

van Zijderveld, S. M., Fonken, B., Dijkstra, J., Gerrits, W. J. J., Perdok, H. B., Fokkink, W., & Newbold, J. R. (2011). Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. *Journal of Dairy Science, 94*(3), 1445-1454. doi: 10.3168/jds.2010-3635

van Zijderveld, S. M., Gerrits, W. J. J., Apajalahti, J. A., Newbold, J. R., Dijkstra, J., Leng, R. A., & Perdok, H. B. (2010). Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *Journal of Dairy Science, 93*(12), 5856-5866. doi: 10.3168/jds.2010-3281

van Zijderveld, S. M., Gerrits, W. J. J., Dijkstra, J., Newbold, J. R., Hulshof, R. B. A., & Perdok, H. B. (2011). Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *Journal of Dairy Science, 94*(8), 4028-4038. doi: 10.3168/jds.2011-4236

Waghorn, G. C., & Hegarty, R.S. (2011). Lowering ruminant methane emissions through improved feed conversion efficiency. Animal Feed Science and Technology, 166-167(0), 291-301. doi:http://dx.org/10.1016/j.anifeedsci.2011.04.019

Whitford, M. F., Teather, R. M., & Forster, R. J. (2001). Phylogenetic analysis of methanogens from the bovine rumen. *BMC microbiology, 1*, 5. doi: 10.1186/1471-2180-1-5

Woldeghebriel, A., Smith, S., Barrios, T., & Gebrelul, S. (2013). Impacts of high fiber diet on digestion and absorption of minerals in growing pigs. *Journal of Agricultural Science and Technology, 2013,* 886–895.

Wolin, M. J. (1960). A theoretical rumen fermentation balance. *Journal of Dairy Science, 43*(10), 1452-1459. doi: 10.3168/jds.S0022-0302(60)90348-9

Wood, T. A., Wallace, R. J., Rowe, A., Price, J., Yáñez-Ruiz, D. R., Murray, P., & Newbold, C. J. (2009). Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. *Animal Feed Science and Technology, 152*(1-2), 62-71. doi: 10.1016/j.anifeedsci.2009.03.006

Yan, T., Mayne, C.S., & Porter, M.G. (2006). Effects of dietary and animal factors on methane production in dairy cows offered grass silage-based diets. International Congress Series, 1293(0), 123-126. doi:http://dx.doi.org/10.1016/j.jcs.2006.02.024

Yan, T., Mayne, C. S., Gordon, F. G., Porter, M. G., Agnew, R. E., Patterson, D. C., . . . Kilpatrick, D. J. (2010). Mitigation of enteric methane emissions through improving

efficiency of energy utilization and productivity in lactating dairy cows. *Journal of Dairy Science, 93*(6), 2630-2638. doi: 10.3168/jds.2009-2929

Yang, C. J., Mao, S. Y., Long, L. M., & Zhu, W. Y. (2012). Effect of disodium fumarate on microbial abundance, ruminal fermentation and methane emission in goats under different forage : concentrate ratios. *animal, 6*(11), 1788-1794. doi: doi:10.1017/S1751731112000857

Yu, C. W., Chen, Y. S., Cheng, Y. H., Cheng, Y. S., Yang, C. M. J., & Chang, C. T. (2010). Effects of fumarate on ruminal ammonia accumulation and fiber digestion in vitro and nutrient utilization in dairy does. *Journal of Dairy Science, 93*(2), 701-710. doi: 10.3168/jds.2009-2494

Zebeli, Q., Tafaj, M., Weber, I., Steingass, H., & Drochner, W. (2008). Effects of dietary forage particle size and concentrate level on fermentation profile, in vitro degradation characteristics and concentration of liquid- or solid-associated bacterial mass in the rumen of dairy cows. *Animal Feed Science and Technology, 140*(3–4), 307-325. doi: 10.1016/j.anifeedsci.2007.04.002

Zhou, M., Hernandez-Sanabria, E., & Guan le, L. (2010). Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. *Appl Environ Microbiol, 76*(12), 3776-3786. doi: 10.1128/aem.00010-10

Zhou, M., McAllister, T. A., & Guan, L. L. (2011). Molecular identification of rumen methanogens: Technologies, advances and prospects. *Animal Feed Science and Technology, 166-167*(0), 76-86. doi: 10.1016/j.anifeedsci.2011.04.005

Zhou, Z, Meng, Q., & Yu, Z. (2011). Effects of methanogenic inhibitors on methane production and abundances of methanogens and cellulolytic bacteria in in vitro ruminal cultures. *Applied and Environmental Microbiology, 77*(8), 2634-2639. doi: 10.1128/aem.02779-10

Zhou, Z., Yu, Z., & Meng, Q. (2011). Effects of nitrate on methane production, fermentation, and microbial populations in in vitro ruminal cultures. *Bioresource Technology*(0), 1-7. doi: 10.1016/j.biortech.2011.10.013

Zhou, Y. W., McSweeney, C. S., Wang, J. K., & Liu, J. X. (2012). Effects of disodium fumarate on ruminal fermentation and microbial communities in sheep fed on high-forage diets. *animal, 6*(Special Issue 05), 815-823. doi: doi:10.1017/S1751731111002102

Appendix A: Comparison Study on Methane Prediction Using VFA Concentrations

Introduction

Carbon dioxide and CH_4 are two anthropogenic and naturally occurring GHGs in the atmosphere. The determination and quantification of these gases from ruminant animals has traditionally been carried using tracer and enclosure techniques (Johnson & Johnson, 1995). However enclosure techniques can be expensive and restrictive to the movement of the animal. Therefore, the use of a non-tracer techniques like the sulfur hexafluoride (SF_6) gas technique has been proven to be suitable to measure CH_4 production from grazing animals, but the technique does not account for the production of CH_4 in the hindgut (Boadi & Wittenberg, 2001). The uses of *in vitro* experiments is also a widely accepted approach and allows the researcher to quantitatively estimate gas production in a controlled environment, but the approach can become complex when multiple experiments need to be conducted (Johnson, 1966). Gas production can also be predicted or estimated using equations (Wolin, 1960; Johnson & Johnson, 1995; and Woldeghebriel et al. 2013). The use of mathematical models allows the researcher to assess gas production in a cost effective way without utilizing experimental animals. The correlation between fermentation end products like VFAs and CH_4 production have been emphasized by Boadi et al. (2004) and also VFA concentrations can be a good indicator for the amount of CH_4 produced. The use of models to calculate rumen VFA proportions or CH_4 production have been developed (Wolin, 1960; Morvay et al., 2011) and validated (Blummel et al., 1993). Wolin, (1960) developed a theoretical mathematical equation based on the major VFAs present in rumen fluid. A study published by Woldeghebriel et al. (2013) revealed the stoichiometry equation developed by Wolin, (1960) for ruminant animals could be used to determine the amount of CO_2

and CH_4 produced in growing pigs on low and high fiber diets. Therefore, the stoichiometry equation developed by Wolin, (1960) and later validated by Blummel et al. (1993) could be used to determine the amount of CO_2 and CH_4 produced and to compare the estimated values with the direct measurements of CH_4 production in the current study.

The Mathematical Model

The stoichiometrical equations developed by Wolin, 1960 were used to determine CO_2 and CH_4 concentrations using the mean VFA concentrations from experiment 1. The model is based on the assumption that simple sugars are converted into the three major VFAs acetate, butyrate, and propionate (Wolin, 1960). The mean concentrations of VFAs were obtained and used to calculate the concentrations of CO_2 (Eq. 5) and CH_4 (Eq. 6) as follows:

$$CO_2\,(mol) = A/2 + P/4 + 1.5B \quad \text{(Eq. 9)}$$

$$CH4\,(mol) = (A + 2B) - CO_2 \quad \text{(Eq. 10)}$$

(Where A, P and B are moles of acetate, propionate and butyrate, CH_4 moles of methane and where CO_2 moles of carbon dioxide estimate in Eq. 5).

Results

Table 17

Concentrations of CH_4 in ppm

Animal	PS, mm	Control	Nitrate	Fumarate	Nitrate-Fumarate	SEM
Cow	S	770.8^{a}	642.3^{b}	677.4^{b}	897.7^{c}	57.7
	M	600.2^{a}	378.8^{b}	654.8^{a}	1044.1^{c}	138.4
	L	832.3^{a}	354.5^{b}	683.7^{c}	340.5^{b}	122.3
Steer	S	455.2^{a}	353.9^{b}	381.1^{c}	391.1^{c}	21.5
	M	322.6^{a}	225.6^{b}	303.8^{a}	284.0^{a}	21.0
	L	965.1^{a}	231.2^{b}	803.6^{c}	688.2^{c}	157.5

Note. Different superscripts with in a row indicates means that are different ($p < 0.05$)

Table 17 shows the concentration of CH_4 determined using the VFA concentrations from the steer and cow by particle size in experiment 1. Addition of nitrate consistently reduced CH_4 concentration for all PS compared to the control for the cow. Addition of fumarate also decreased CH_4 concentrations for the small and large PS, while fumarate had no effect on the medium PS compared to the control. However there was no significant difference between the effects of addition of fumarate and nitrate for the small PS. Addition of the nitrate-fumarate combination increased CH_4 concentrations for the small and medium PS for the cow compared to control, nitrate or fumarate. The addition of the nitrate-fumarate combination also reduced CH_4 concentrations for the large PS compared to fumarate or the control but with no significant difference from nitrate.

The addition of nitrate also reduced CH_4 concentrations compared to the control, fumarate or nitrate-fumarate combination for the steer. Addition of fumarate also reduced CH_4 concentrations for the small and large PS, but had no effect on the medium PS compared to the control. Addition of the nitrate-fumarate combination reduced CH_4 concentrations for the small and large PS, but no significant effect for the medium PS compared to the control. There was also no significant difference between the fumarate and the nitrate-fumarate combination for all PS.

In summary, addition of nitrate consistently reduced CH_4 concentrations in both animals and all PS. The addition of fumarate also reduced CH_4 concentrations for the small and large PS but with no significant effect for the medium PS. The nitrate-fumarate combination had variable effects on CH_4 production. For example, the model estimated an increase in CH_4 production for the small and medium PS with a corresponding decrease for the large PS from the cow. It was

also noted that the nitrate-fumarate combination reduced CH_4 concentrations for the small and large PS with no significant effect for the medium PS from the steer.

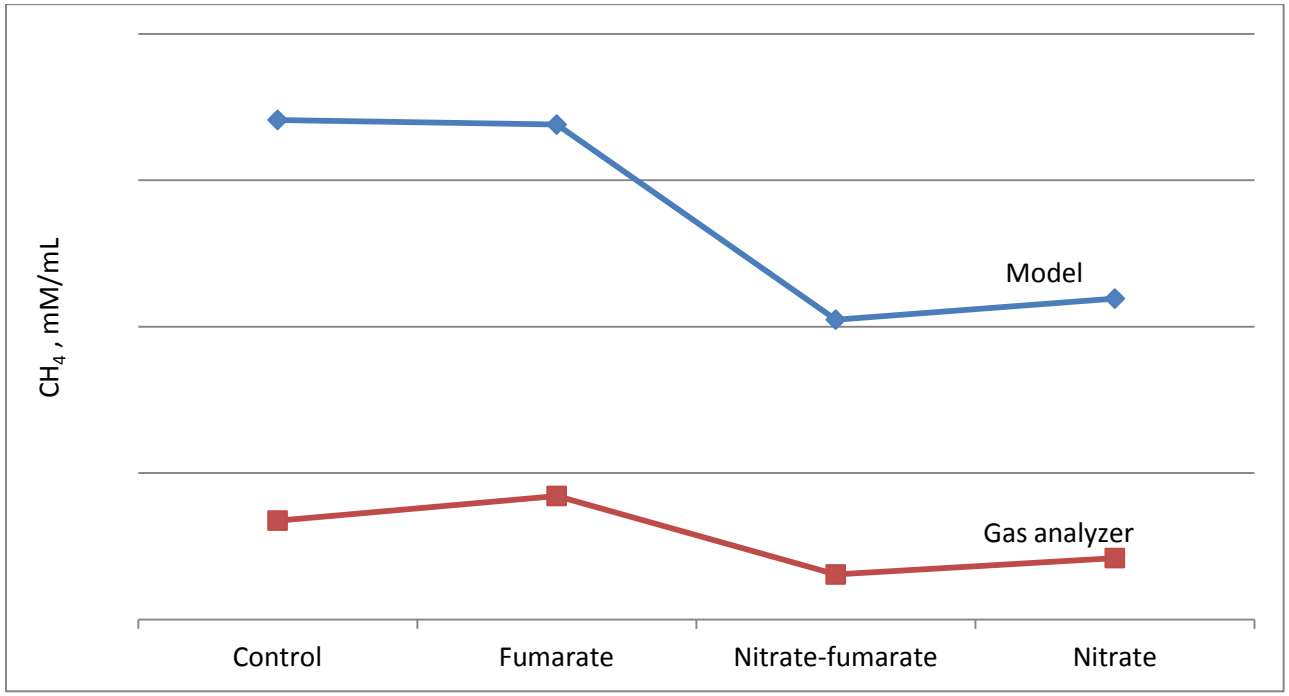

Figure 14. Comparison of CH_4 Production from the Model with the Actual Measurement Using the Gas Analyzer

Figure 14 shows the concentration of CH_4 from the model and the gas analyzer. Overall, the model overestimated CH_4 production compared to the direct measurement by the gas analyzer. Even though the model showed that addition of nitrate alone or in combination with fumarate effectively lowered CH_4 concentrations compare to the addition of fumarate or the control the model clearly overestimated the concentration CH_4 compared to the gas analyzer. However, the model showed a trend similar to the gas analyzer even though there were quantitative differences between them.

Summary and Discussion

The formation of short chain fatty acids like acetate, butyrate and propionate along with gases like CO_2 and CH_4 are all characteristics of microbial feed degradation (Blummel et al., 2005). However, CH_4 is produced during the synthesis of acetate and butyrate, but not during propionate synthesis (Wolin, 1960). The model developed by Wolin (1960) made the assumption that the sole fermentation end products are the three major VFAs, CO_2, and CH_4 with hexose as the fermentation substrate. Therefore, it is based on that assumption that the estimated CH_4 concentrations were determined from the three major VFAs (acetate, propionate, and butyrate) produced in the current study. Even though it was not clear why, it was interesting to learn that both the model and the gas analyzer showed that the medium PS had the lowest concentration of CH_4. The results obtained in experiment 1 also revealed that CH_4 production for the medium PS was 31% lower than the small PS and 39% lower than the large PS, while the estimated values from the model indicated that CH_4 production for the medium PS was only 22% lower than the small PS and 28% lower than the large PS. It was also estimated that nitrate consistently reduced CH_4 production for the cow and the steer compared to the control and fumarate. The addition of nitrate reduced all *in vitro* individual VFA production. Therefore, a decrease in two of the three major VFAs acetate and butyrate could be responsible for the decrease in the estimated CH_4 concentrations which is line with the work of Wolin, (1960); Blummel et al. (1993); and Alemu et al. (2011) who reported that a decrease in acetate can reduce methane concentrations. The use of fumarate had no effect on the actual measurements of CH_4 concentrations, but the model estimated up to 18% reduction in CH_4 production and this could possibly be explained by the fact that addition of fumarate increased propionate production compared to the control in the current tudy. The model estimated that addition of the nitrate-fumarate combination had variable effects

on CH_4 production. For instance, for the cow the nitrate-fumarate combination decreased CH_4 production for the large PS, but increased for the small and medium PS compared to the control. However, in the steer the nitrate-fumarate combination reduced concentration of CH_4 for the small and large PS, but had no effect for the medium PS compared to the control. This was not in agreement with the measurements from experiment 1 where the nitrate-fumarate combination reduced CH_4 production. The inability of the model to closely estimate CH_4 production using the three major VFAs could be due to the fact that rumen pH, microbial populations, and other pathways that uptake H_2 (Blummel et al., 1997 and Alemu et al., 2011) were not considered as factors that can directly or indirectly affect enteric CH_4 production.

Conclusion

In conclusion using the stoichiometry equation developed for ruminant animals we were able to predict a trend similar to the direct measurement of CH_4 production using the gas analyzer. However, the model overestimated the reduction of CH_4 production by the addition of fumarate and underestimated the reduction of CH_4 production by nitrate. The combined effect of nitrate and fumarate did not seem to show any consistent reduction of CH_4 production. Therefore, while the model effectively estimated the effects of treatments on *in vitro* methane production its ability to generate testable data close to the actual measurements of methane concentrations seem to need further improvement.

Appendix B: Estimated CO_2 Concentrations

Table 18

CO_2 Concentrations in ppm

Cow	PS, mm					
Treatment	0.85	±SE	1.4	±SE	2.36	±SE
Control	1413.6[a]	127.7	1184.2[a]	347.1	1586.4[a]	264.3
Nitrate	1021.7[b]	127.7	594.8[b]	347.1	540.4[b]	264.3
Fumarate	1330.2[a]	127.7	1401.8[a]	347.1	1369.0[a]	264.3
Nitrate-Fumarate	1639.1[c]	127.7	2269.6[c]	347.1	612.83[c]	264.3

Different superscripts in a column are means that are different ($p < 0.05$)

Table 19

CO_2 Concentrations in ppm

Steer	PS, mm					
Treatment	0.85	±SE	1.4	±SE	2.36	±SE
Control	951.3[a]	76.6	663.0[a]	68.3	1935.8[a]	357.4
Nitrate	586.9[b]	76.6	369.2[b]	68.3	376.2[b]	357.4
Fumarate	838.7[c]	76.6	630.2[a]	68.3	1844.7[c]	357.4
Nitrate-Fumarate	759.6[c]	76.6	628.8[a]	68.3	1361.5[b]	357.4

Different superscripts in a column are means that are different ($p < 0.05$)

.

Appendix C: DNA Sequences and BLASTn Results

Table 20

Summary of Sequence Comparison using the NCBI BLAST tool

Accession	Description	Max Score	Query Coverage	E value	Max Ident
NC 007681.1	Methanosphaera stadtmanae DSM 3091	183	89%	2e-44	93%
NC013790.1	Methanobrevibacter ruminantium M1	148	82%	1e-33	90%
JQ728441.1	Uncultured Methanosphaera Sp. Clone 6cL 16S ribosomal RNA gene	64.4	32%	3e-07	86%
NC009515.1	Methanobrevibacter Smithii ATCC 35061	189	74%	6e-46	98%