INVESTIGATING THE POTENTIAL OF SUPPLEMENTARY NITRATE AND MONENSIN AS DIETARY ADDITIVES FOR ENTERIC METHANE MITIGATION IN RUMINANTS

By

Matheus Gabriel Maidana Capelari

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Animal Science – Doctor of Philosophy

2018

ABSTRACT

INVESTIGATING THE POTENTIAL OF SUPPLEMENTARY NITRATE AND MONENSIN AS DIETARY ADDITIVES FOR ENTERIC METHANE MITIGATION IN RUMINANTS

By

Matheus Gabriel Maidana Capelari

Enteric CH_4 production from ruminant livestock is the largest source of anthropogenic CH_4 emissions in the United States and in most countries with significant livestock production. Because animal products from ruminants are an important part of the diet and CH_4 from ruminant animals contributes to greenhouse gas (GHG) emissions, finding ways to reduce CH_4 from the livestock production are essential to long-term viability of ecosystems and consumer food choices. In addition, CH_4 represents a significant dietary GE loss and reduction in methanogenesis can result in improved feed utilization. Supplementing the diets of ruminants with nitrate salts consistently decrease CH_4 production without signs of microbial adaptation in the long-term. Nitrate reduction is a biochemical process that consumes H_2, which, in the rumen, is the substrate used by methanogenic archaea for energy production. Monensin sodium has been extensively used in ruminant production because it increases feed conversion and the reduction in CH_4 production from monensin feeding is mainly related with more efficient fermentation and reduced protozoa numbers. Therefore, nitrate and monensin affect methanogenesis through different mechanisms. The objective of this study was to evaluate if the combination of nitrate and monensin could additively reduce CH_4 production without compromising animal health and performance. A series of experiments were conducted to test the effect of additives on enteric CH_4 production and ruminal fermentation parameters. Independent of diet composition, nitrate and monensin additively reduced CH_4 production with *in vitro* models, with maximum reduction of approximately 90% on batch culture trials and 45% on semi-continuous culture fermenters. In

batch culture trials the combination of additives at higher doses (2.5% of nitrate in the diet and 6 mg of MON per L of culture media) reduced digestion of feeds. In the semi-continuous fermenters a similar effect on nutrient digestion was observed when additives were combined in higher levels tested (2.5% of nitrate in the diet and 4 mg of monensin per L of culture media), but no effect was observed on VFA production. An intermediary dose of nitrate (1.5% of DM) was tested in growing steers and a significant reduction of 10% for CH_4 production was observed. Animals fed nitrate did not demonstrate any clinical sign of toxicity during the experiment with methemoglobin levels similar to control animals. Monensin reduced CH_4 production by growing steers by 5% when compared to control. When combined, additives reduced CH_4 production by approximately 16% without affecting ruminal fermentation, demonstrating a potential for utilization of additives in the diet of ruminants as a means of CH_4 abatement. Long-term performance trials coupled with CH_4 measurements are needed to confirm that this strategy does not impair animal performance and are able to sustain reduction on CH_4 levels for longer periods of time.

ACKNOWLEDGMENTS

I have many special people to thank. I would first like to thank my advisor Dr. Wendy Powers for accepting me in your group and for being supportive and patient with me throughout these years. I did not come to the Arctic because I enjoy snow, but because I knew you would be a great mentor and the scientist I needed to guide me. I would also like to thank my guidance committee members: Dr. Kristen Johnson, Dr. Wei Liao, Dr. Steven Rust and Dr. Mike Allen. Thank you very much for always keeping your office and labs doors open for discussions and analytical work.

I would like to thank my lab mates that during these years at MSU helped me through intensive experimental procedures. Jolene Roth, Broke Latack and Mark Schilling I will always reserve a special part of my dissertation acknowledgements for you. Thank you for the patience to teach me the procedures and tasks needed and for helping me with sample collection and analysis. I would also like to thank all the 14 undergraduate students who helped me during this time. Without the hard work of these students I would not have been able to collect and analyze samples, prepare materials needed for the experiment and manage the animals used in the trials. I would also like to thank lab technicians from the animal science department who were also super important. Special thanks to Dave Main who became a great friend and taught me a bunch on feed analysis. A special thank also to Jane Link and Jim Liesman who were great friends and always helpful when I needed.

Last but not least I would like to express my deepest gratitude and love for my brother Mauro Guilherme, and specially my parents Marisa Maidana and Mauro Capelari, who always

supported and prioritize our education in detriment of their own life. This degree is mainly dedicated to both of you.

TABLE OF CONTENTS

LIST OF TABLES…………………………………………………………………………….. viii

KEY TO ABBREVIATIONS………………………………………………………………… x

CHAPTER 1. LITERATURE REVIEW………………………………………………………. 1
1.1. The ruminant animal and its role in human nutrition and evolution ………………… 2
1.2. Methanogenesis in anaerobic environments…………………………………………... 4
1.3. Fermentation and enteric methanogenesis in ruminants ……………………………… 6
1.4. Ruminants and global warming …………………………………………………… 7
1.5. Strategies to reduce enteric methane emissions……………………………………….. 9
1.5.1. Nutrition………………………………………………………………... 9
1.5.2. Biotechnology…………………………………………………………….. 15
1.5.3. Genetic selection………………………………………………………….. 15
1.6. Using nitrate to reduce enteric methane emissions……………………………………. 17
1.6.1. Effect of nitrate on enteric methane emission……………………………… 17
1.6.2. Effects of nitrate on production parameters……………………………….... 19
1.7. Using monensin to reduce enteric methane emissions……………………………….... 21
1.8. Objectives and rationale………………………………………………………………….. 23
LITERATURE CITED ……………………………………………………………………. 25

CHAPTER 2. THE EFFECT OF NITRATE AND MONENSIN ON *IN VITRO* RUMINAL FERMENTATION ……………………………………………………………………….. 38
2.1. Abstract……………………………………………………………………….…...... 40
2.2. Introduction…………………………………………………………………………. 41
2.3. Material and Methods…………………………………………………………………. 42
2.3.1. Experimental design and treatments……………………………………... 42
2.3.2. Incubation conditions and rumen inoculum source……………………….... 43
2.3.3. Sampling and measurement procedures…………………………………… 44
2.3.4. Fermentation balance calculations………………………………………….. 46
2.3.5. IVDMD……………………………………………………………………... 47
2.3.6. Statistical analysis………………………………………………………….... 48
2.4. Results……………………………………………………………………………… 48
2.4.1. EXP1 (100:0 roughage:concentrate)…………………………………….... 49
2.4.2. EXP2 (10:90 roughage:concentrate)…………………………………….... 50
2.5. Discussion………………………………………………………………………….... 50
2.5.1. CH_4, N_2O and VFA production………………………………………… 54
2.5.2. Fermentation balance………………………………………………………. 58
2.5.3. IVDMD……………………………………………………………………….. 55
2.5.4. The potential of using nitrate as feed additive for ruminants……………… 57
2.6. Conclusion…………………………………………………………………………... 58
LITERATURE CITED………………………………………………...……………... 69

CHAPTER 3. THE EFFECT OF TWO ADDITIVES ON RUMINAL ERMENTATION USING A SEMI-CONTINOUS CULTURE SYSTEM………………………………………………….. 75
3.1. Abstract…………………………………………………………………………..... 77
3.2. Introduction……………………………………………………………………….. 78
3.3. Material and Methods…………………………………………………………….... 79
3.3.1. Experimental design and treatments……………………………………… 80
3.3.2. Rumen Simulation System……………………………………………….. 81
3.3.3. Incubation procedure and initial experimental procedures………………...... 82
3.3.4. Sampling and analytical procedure……………………………………… 83
3.3.5. Fermentation balance calculations………………………………………... 85
3.3.6. Statistical analysis………………………………………………………… 86
3.4. Results…………………………………………………………………………...... 87
3.4.1. DAIRY trials (50:50 roughage:concentrate)……………………………….. 87
3.4.2. BEEF trials (10:90 roughage:concentrate)……………………………….. 88
3.5. Discussion……………………………………………………………………….... 89
3.5.1. Fermentation products……………………………………………………. 89
3.5.2. Fermentation balance……………………………………………………...... 94
3.5.3. Nutrient digestion………………………………………………………… 95
3.6. Conclusion………………………………………………………………………… 97
LITERATURE CITED……………………………………...…………………… 114

CHAPTER 4. THE EFFECT OF NITRATE AND MONENSIN ON RUMEN FERMENTATION AND ENTERIC METHANE EMISSION OF GROWING BEEF STEERS…………………………………………………………………………...... 116
4.1. Abstract…………………………………………………………………………… 117
4.2. Introduction……………………………………………………………………...... 118
4.3. Material and Methods…………………………………………………………….... 118
4.3.1. Animals, diet and experimental design…………………………………....... 119
4.3.2. Sampling and measurement procedure………………………………........ 122
4.3.3. Laboratory analysis……………………………………………………… 123
4.3.4. Statistical analysis……………………………………………………....... 123
4.4. Results…………………………………………………………………………...... 124
4.5. Discussion…………………………………………………………………………. 123
4.5.1. Nitrate Toxicity…………………………………………………………....... 126
4.5.2. Methane emissions……………………………………………………….... 128
4.5.3. Rumen fermentation profile……………………………………………… 129
4.5.4. Nitrate as a Feed Additive for Ruminants…………………………………... 130
LITERATURE CITED……………...…………………………………………… 127

CHAPTER 5. SUMMARY……………………………………………………………... 143

LIST OF TABLES

Table 2.1. Ingredients and chemical composition of experimental diets for *in vitro* EXP1 (100:0 roughage: concentrate) and EXP2 (10:90 roughage: concentrate)[1]………………………………......... 59

Table 2.2. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* gas and CH_4 production in EXP1 (100:0 roughage:concentrate... 61

Table 2.3. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* VFA profile and fermentation balance in EXP1 (100:0 roughage:concentrate)[1]………………………………………………………………………… 63

Table 2.4. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* gas and CH_4 production in EXP2 (10:90 roughage:concentrate)……………………………………………………………………….. 65

Table 2.5. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* VFA profile and fermentation balance in EXP2 (10:90 roughage:concentrate)[1]………………………………………………………………………… 67

Table 3.1. Ingredients and chemical composition of basal diets for DAIRY (50:50 roughage: concentrate) and BEEF (15:85 roughage: concentrate) trials……………………………………… 98

Table 3.2. Composition of experimental treatments of the Rusitec DAIRY (50:50 roughage: concentrate) and BEEF (15:85 roughage: concentrate) trials………………………………........... 99

Table 3.3. Effects of EN and MON on gas production and nutrient disappearance on DAIRY (50:50 roughage: concentrate) trials…………………………………………………............. 100

Table 3.4. Effects of ENP and MON on fermentation parameters on DAIRY (50:50 roughage: concentrate) trials……………………………………………………………………….......... 101

Table 3.5. Effects of EN and MON on gas production and nutrient disappearance on BEEF (50:50 roughage: concentrate) trials…………………………………………………............. 103

Table 3.6. Effect of EN and MON on rumen fermentation parameters of BEEF (15:85 roughage: concentrate) trials……………………………………………………………………….. 104

Table 4.1. Feed ingredients and chemical composition of the diets……………………………… 131

Table 4.2. Effects of nitrate (NIT), monensin (MON) and the combination of additives on BW gain and blood hemoglobin and methemoglobin levels of steers………………………………… 133

Table 4.3. Effects of nitrate (NIT) and sodium monensin (MON) on enteric CH_4 emissions and CH_4 yield………………………………..……………………………………………………… 134

Table 4.4. Effect of nitrate (NIT) and sodium monensin (MON) on rumen fermentation profile of steers……..………………………………………………………………………………..135

KEY TO ABBREVIATIONS

AAQRF.. Animal Air Quality Research Facility

ADG .... average daily gain

ADF.. acid detergent fiber

A:P .... acetate to propionate ratio

BCTRC .... Beef Cattle Teaching Research Facility

BW .... body weight

C .... carbon

CH_4 .... methane

CO_2Eq.. CO_2 equivalent

CP .... crude protein

DE .... digestible energy

DM .... dry matter

DMI .... dry matter intake

ECD .... electron capture detector

F:C .... forage to concentrate ratio

FID .... flame ionization detector

GC .... gas chromatograph

GEI .... gross energy intake

GHG... greenhouse gas

GE.. gross energy

H_2 .... hydrogen gas

IVDMD .... *in vitro* dry matter digestibility

N .... nitrogen

N_2 .... nitrogen gas

N_2O .... nitrous oxide

NH_3 .... ammonia

NDF .... neutral detergent fiber

NFC .... non-forage carbohydrate

NPN .... non-protein nitrogen

CHAPTER 1. LITERATURE REVIEW

1.1. The ruminant animal and its role in human nutrition and evolution

Ruminants can be distinguished from other mammalian species by their highly specialized, compartmentalized stomach system. The largest of the four compartments is called the rumen, which hosts a symbiotic population of microflora and fauna that secretes enzymes allowing for the breakdown of structural cell wall components of plants that otherwise would not be digested. Following the breakdown of polysaccharides, microbes quickly absorb and ferment simple sugars (mainly hexose and pentoses) and amino acids, yielding energy and nitrogen necessary for microbial growth, and by-products such as volatile fatty acids and gases (mainly CO_2 and CH_4, and to a less extent NH_3 and H_2). The VFA are passively absorbed from the rumen into the blood stream and are the primary energy source for the animal. Likewise, microbial biomass forms a valuable source of protein to the animal as it flows out of the rumen to be digested in the small intestine. Gases that have been formed are eructated and exhaled by the host animal. The ability to utilize plant cell wall allows ruminants to survive on substrates inaccessible to humans and has allowed the ruminant to occupy a specific niche in the animal kingdom (Van Soest, 1994).

The importance of ruminants for human history can be divided into two main events that directly shaped the evolution of the specie: 1) the inclusion of meat in the diet of early *Homo* species, and 2) the advent of agriculture and domestication of ruminant species and plants.

The inclusion of meat protein to a previous plant based diet of early *Homo* (Wood and Collard (1999) argue that *Homo ergaster*, early African *Homo* erectus, diverged from *Australopithecus* between 1.9 and 1.5 million years ago) increased energy density and overall diet quality compared with other primates (Aiello and Wells, 2002). Meat protein is easier to digest than plant protein and even with a limited amount of fat would still have been a valuable source of essential amino acids, fatty acids, fat soluble vitamins and minerals (Milton, 1999). An

added advantage of including meat in the diet is the high methionine content of animal protein, which provides an adequate supply of sulfur-containing amino acids necessary for detoxification of toxic (cyanogenic) plant foods (Milton, 1999). The diet with increased meat protein supported the development of high energy cost neural tissue, especially during childhood when a rapid expansion of brain size occurs. In addition, an increased dependence on hunting and meat scavenging imposed challenges to early *Homo*, such as competition with other carnivores and long training necessary for skill development, a setting that stimulated the learning of social rules and more sophisticated communication. The combination of dietary changes with a more social environment are thought to be major selective drivers of larger brains and were main determinants on human evolution (Hawks, 2016).

The domestication of one species by another for food is one of the most significant evolutionary innovations in the history of life on the planet (Cavanaugh and Currie, 2016) and the advent of agriculture is argued to be the most important force shaping human history (Diamond, 1997). In fact, as much as it shaped human society and history, it has also had an important role in the development of evolutionary theory, as evident in Darwin's *On the Origin of Species*, which begins with a through discussion of domestication of animals and plants – an evolutionary force he termed "artificial selection" – even before introducing tenets of natural selection:

> "It is…of the highest importance to gain a clear insight into the means of modification and coadaptation. At the commencement of my observations it seemed to me probable that a careful study of domesticated animals and of cultivated plants would offer the best chance of making out this obscure problem. Nor have I been disappointed; in this and in all other perplexing cases I have

invariably found that our knowledge, imperfect though it be, of variation under domestication, afforded the best and safest clue (1859)."

Although the first ruminant species evolved around 50 million years ago, ruminant domestication started more recently, about 10,000 years ago, along with the advent of agriculture. Initially, ruminants were used primarily for meat production with the husbandry of goats (Zeder and Hesse, 2000), but later ruminants were also used for milk production, draught power, transportation, currency and in religious rituals (Clutton-Brock, 1999). Today, global population of domesticated ruminants is estimated to be around 3.6 billion, nearly 50 times greater than the wild species population (Hackmann and Spain, 2010).

Ruminants have a strategic position relative to man by deriving food from fiber and non-protein resources without competing with man for food (Van Soest, 1994; White and Hall, 2017). Nowadays, ruminants play a major role in human nutrition. They are the predominant source of milk (816 million metric tons in 2016; OECD-FAO, 2017) and supplied approximately 26% of the global meat for human consumption in 2016 (68.471 and 14.318 Kt carcass weight equivalent from beef and sheep, respectively; OECD-FAO, 2017). Although per capita milk and meat consumption have stabilized in developed countries, economic growth in developing countries will drive increased demand for ruminants products in the next 30 years (Steinfield et al., 2006).

1.2. Methanogenesis in anaerobic environments

The biological production of methane (CH_4) – methanogenesis – is carried out by a group of strictly anaerobic archaea called methanogens. Phylogenetically, archaea have a distinct evolutionary history compared to bacteria, and physiologically, the composition of cellular constituents are significantly different. The characteristic peptidoglycan polymer of the cell walls

of bacteria is absent from methanogens and other archaea. Archaea lipids are glycerol ethers rather than glycerol esters. In addition, archaea chromosomes are circular and the translation and transcription processes are more similar to eukarya than bacteria. Overall, most of the metabolic pathways are common between archaea and bacteria, while most genes involved in genome expression are common between archaea and eukarya. Within methanogens, although many common physiological features are shared, considerable phylogenetic diversity exists and is reflected in the representation of morphologies (e.g. cocci, various shaped rods, spirilla), environmental adaptations (e.g.thermophilic and mesophilic) and motility ability of different genera and species of methanogens (Madigan et al., 2006).

At least 11 substrates have been shown to be converted to CH_4 by pure cultures of methanogens. With the appropriate mixed culture, virtually any organic compound, including hydrocarbons, can be converted to CH_4 plus CO_2 (Madigan et al., 2006). The main substrates for methanogenesis can be divided into 3 classes: 1) CO_2-type substrates, which include CO_2 itself, formate and carbon monoxide, 2) methyl substrates, which includes methanol, methylamine and dimethylamine, and 3) acetotrophic substrates, such as acetate and pyruvate. This flexibility provides methanogens the ability to survive in different environments where CH_4 formation has been observed such as 1) anoxic sediments in marsh, swamp, lakes, paddy fields and moist landfills (Lyimo et al., 2002), 2) digestive tract of herbivore animals such as ruminants (Hungate, 1966), hindgut of horses (Dansen et al., 2015) and rabbits (Franz et al., 2011), large intestine of monogastrics such as swine (Li et al., 2011) and humans (Kunkel et al., 2011), 3) geothermal sources of H_2 and CO_2 such as hydrothermal vents (Etiope et al., 2007), 4) artificial biodegradation facilities for sewage sludge digestion (Yoshida et al., 2011), and 5) performing endosymbiosis with various anaerobic protozoa (Guyader et al., 2014).

The biological production of CH_4 occurs through a series of reactions involving novel coenzymes and amazing complexity. The key coenzymes can be divided into 1) those involved in carrying the C unit from initial substrate to the final product (e.g. methanofuran, methanopterin, coenzyme M and coenzyme F_{430}), and 2) those that function in redox reactions to supply the electrons needed for the reduction of CO_2 to CH_4 (e.g. coenzyme F_{420}, methyl reductase and coenzyme B). Energy conservation during CH_4 formation is linked to the terminal step, a reduction carried out by methyl reductase enzyme, with extrusion of protons across membrane and creation of a proton motive force (Madigan et al., 2006).

1.3. Fermentation and enteric methanogenesis in ruminants

Volatile fatty acids, principally acetate, propionate and butyrate but also lesser amounts of valerate, caproate, isobutyrate, isovalerate, 2-methylbutyrate and traces of various higher acids, are produced in the rumen as end-products of microbial fermentation (Dijkstra et at al., 2005). For microbes VFA are waste products, but to the host animal they represent the major source of absorbed energy; approximately 80% of the energy disappearing in the rumen for most diets with the remainder being lost as heat and CH_4. In the rumen, CH_4 formation is an evolutionary mechanism for disposal of H_2 generated during anaerobic fermentation of organic compounds. This process allows reduced cofactors to be re-oxidized, thereby avoiding accumulation of H_2 which can impair fermentation. During glucose oxidation, NAD^+ is reduced to $NADH^+$ and reoxidation of $NADH^+$ to NAD^+ is necessary for continued fermentation (Moss et al., 2000; Mc Allister and Newbold., 2008). Most CH_4 is formed in the rumen from H_2 and CO_2 (equation 1), but other potential substrates include formate, acetate, methylamine and methanol (Hungate, 1966). The stoichiometry of production of the main VFA determines the amounts of CO_2 and CH_4 associated with the formation of acetate, propionate and butyrate, the three major VFA

produced in the rumen. Acetate formation releases 2 moles of CO_2 and 4 moles of H_2 per mole of glucose fermented (Equation 2). Four moles of H_2 can then be used by methanogens to reduce 1 mole of CO_2 to CH_4. Therefore, acetate formation results in the production of 1 mole of CH_4 and 1 mole of CO_2 per mole of glucose fermented. Similarly, butyrate production results in 1.5 moles of CO_2 and 0.5 moles of CH_4 per mole of glucose fermented (Equation 3). However, propionate formation requires a net input of H_2, resulting in decreased CH_4 production (Equation 4). Because of the differences in the stoichiometries of CH_4 production relative to VFA formation, the proportion of energy from glucose retained as acetate, propionate and butyrate are 0.62, 1.09 and 0.78, respectively.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{(Equation 1)}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2^- + 2CO_2 + 4H_2 + 2H^+ \qquad \text{(Equation 2)}$$

$$C_6H_{12}O_6 \rightarrow C_4H_7O_2^- + H^+ + 2H_2 + 2CO_2 \qquad \text{(Equation 3)}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2^- + 2H^+ + 2H_2O \qquad \text{(Equation 4)}$$

1.4. Ruminants and global warming

Human activities, especially emissions of GHG, are significant causes of the observed warming since the mid-20th century (IPCC, 2014; Wuebbles et al., 2017). Anthropogenic GHG emissions have increased since the pre-industrial era, driven largely by economic and population growth, and are now higher than ever. This has led to atmospheric concentrations of CO_2, CH_4 and N_2O that are unprecedented over the last 800,000 years. The effects of increased atmospheric concentration of GHG, together with those of other anthropogenic drivers, have been detected throughout the climate system and are the dominant cause of the observed warming since the mid-20th century. Changes in many extreme weather and climate events have been observed

since about 1950. Some of these changes have been linked to human influences, including a decrease in cold temperature extremes, an increase in warm temperature extremes, an increase in extreme high sea levels and an increase in the number of heavy precipitation events in a number of regions (IPCC, 2014).

Among the main anthropogenic sources of GHG are the energy sector, industrial activities and agriculture (Steinfeld et al., 2006). Animal agriculture contributes significantly to the emissions of CO_2, CH_4 and N_2O, with greater concern about CH_4 and N_2O (Powers et al., 2014). These 2 gases have a 100-year global warming potential (GWP) of 25 and 298 (ability to absorb radiation relative to CO_2 (GWP = 1), respectively (IPCC, 2007). Globally, livestock contribute 6.3% of total anthropogenic GHG emissions; CH_4 emissions from enteric fermentation account for 2.1 Gt CO_2 Eq/yr and manure management accounts for 0.99 Gt CO_2 Eq/yr (IPCC, 2014).

Examining CH_4 emissions from livestock agriculture as a proportion of total anthropogenic GHG emissions in a given country or region can be misleading. Methane emissions are a function of the population of ruminant animals, their level of production, and the associated manure-handling systems (Knaap et al., 2014). When assessing ruminant CH_4 emissions by country, it is clear that emissions vary substantially, due mainly to levels of technology used in the system (Thorpe, 2009). In the United States, enteric fermentation of feed by ruminants is the largest source of anthropogenic CH_4 emissions with about 140 million metric tons of CO_2-eq (25% of the total CH_4 emissions; EPA, 2014). In the EU, approximately 66% of total CH_4 emissions have been attributed to enteric fermentation of ruminants, which represents approximately 170 million tons of CO_2-eq (Moss et al., 2000). However, in developed countries where pastoral agriculture is a significant portion of the economy (e.g., Ireland and New Zealand) or developing countries with large cattle populations (e.g., Brazil and India), ruminant

livestock can be a very large contributor to the national GHG inventory (Tubiello et al., 2015). In New Zealand, enteric CH_4 represents approximately 86% of anthropogenic CH_4 emissions (Robertson and Waghorn, 2002). In Brazil, with a production system characterized by pasture based feeding systems and low technology input, CH_4 emission from enteric fermentation totaled 278 million tons of CO_2-eq in 2006（93% of agricultural emissions and 72 % of the country total emissions of CH_4; Ministerio da Ciencia e Tecnologia – Embrapa report, 2010).

1.5. Strategies to reduce enteric methane emissions

In the last few years, many reviews on the different strategies to mitigate enteric CH_4 production by ruminants have been published (i.e. Moss et al., 2000; McAllister and Newbold, 2008; Martin et al., 2010; Hristov et al., 2013; Knaap et al., 2014). Strategies can be summarized into 3 main areas: 1) nutrition, 2) biotechnology, and 3) genetics (animal phenotype) selection.

1.5.1. Nutrition

Among those 3 main categories, nutritional strategies are more developed and ready for field application (Morgavi et al., 2010). As previously discussed, the production and utilization of metabolic H_2 in the rumen determines the amount of substrate available for methanogenesis. Therefore, nutritional strategies to reduce CH_4 production should emphasize the production and fate of H_2 and/or the methanogen community number and activity.

Reduction in H_2 production can be achieved by practices that reduce the proportion of acetate and butyrate and increase propionate formation, such as feeding better quality forages, reducing the forage to concentrate ratio in the diet, or through the use of dietary additives. Feed ingredients provide the substrates for microbial fermentation, and differences in feed digestibility and chemical composition alter the amount of energy extracted by the microbes and the patterns of VFA and CH_4 produced (Knaap et al., 2013). Robertson and Waghorn (2002) observed that

CH_4 production from grazing dairy cows increased with forage maturity (from 5% to 6.5% of GE intake in spring and summer, respectively). This is a consequence of increased proportion of cellulose and hemicellulose with a concurrent reduction in the availability of sugars as the plant matures physiologically (Pinares-Patino et al., 2007).

It is well established that increasing the level of concentrate in the diet leads to a reduction in CH_4 emissions as a proportion of energy intake or expressed by unit of animal product (milk and meat; Martin et al., 2010). Replacing structural carbohydrates from forages (cellulose, hemicellulose) in the diet with non-structural carbohydrates (starch and sugars) contained in most energy-rich concentrates is associated with increases in feed intake, higher rates of ruminal fermentation and accelerated feed turnover, which results in large modifications of rumen physico-chemical conditions and microbial populations. A shift of VFA production from acetate towards propionate occurs with the development of starch-fermenting microbes. This results in less CH_4 production because the relative proportion of ruminal H_2 sources declines whereas that of H_2 sinks increases (Martin et al., 2010). A meta-analysis demonstrated that the relationship between concentrate proportion in the diet and CH_4 production is curvilinear (Sauvant and Giger-Reverdin, 2007). Methane losses appear relatively constant for diets containing 30 to 40 percent concentrate (6 to 7 percent of GE intake) and then decrease rapidly to low values (2 to 3 percent of GE intake) for diets containing 80 to 90 percent concentrate (Lovett et al., 2003; Beauchemin and McGinn, 2005; Martin et al., 2007). Even though there is a certain popular perception that grass based, low-input beef and dairy system are more sustainable, the increase in animal productivity observed as a consequence of feeding more digestible diets and through the use of other available technologies (i.e. genetic selection, preventive health programs, improved cow comfort and better management practices) substantially reduces the environmental impact

livestock. The reduction in the environmental impact (total CO_2 equivalent) observed when animal productivity increases is mainly explained by a dilution of maintenance effect (VandeHaar and St-Pierre, 2006), which improves productive efficiency (resource input per unit of food output). The carbon footprint per billion kilograms of milk produced in the United Stated in 2007 was 37% of equivalent milk production in 1944, with 21% of the animals, 23% of feedstuffs, 35% of the water and only 10% of the land required in 1944 (Capper et al., 2009). For beef production, in a study using deterministic models, Capper (2011) observed that, when compared to 1977, modern US system reduced the carbon footprint by 16.3% per billion kilograms of product.

Reduction in CH_4 production by reducing H_2 production can be achieve by feeding additives that control the growth of H_2 generating species. Ionophore antibiotics such as monensin and lasalocid, typically used to improve efficiency of animal production, decrease CH_4 production (reviewed by Beauchemin et al., 2008 and Appuhamy et al., 2013). These ionophores, at the doses prescribed, do not affect methanogens (Chen and Wolin, 1979) but have an inhibitory effect on other microbes, inducing a shift in fermentation towards propionogenesis, the most likely mode of action. This premise is supported by the inability of monensin to reduce CH_4 production when H_2 and CO_2 are provided (Russell and Martin, 1984). Ionophores also affect protozoa; the reduction and subsequent recovery in protozoal numbers perfectly matched CH_4 abatement – up to 30% – and restoration to previous level in a cattle trial (Guan et al., 2006). Essential oils, composed by one, or blends of, plant secondary compounds, have been considered as CH_4 mitigation alternatives, especially after the ban on use of ionophores antibiotics in the European Union (UE) in 2003. A large number of in vitro experiments have investigated the CH_4-mitigating potential of essential oils and their active ingredients

(Calsamiglia et al., 2007; Benchaar et al., 2008). Unfortunately, very few in vitro studies have been followed by in vivo experiments. In most cases, essential oils have not been successful as CH_4 mitigating agents (Beauchemin and McGinn, 2006; Benchaar et al., 2008; Van Zijderveld et al., 2011).

Methane production is reduced if other pathways successfully compete with methanogenesis for the use of H_2 (alternative H_2 sinks) generated during fermentation. This category of CH_4 mitigating agents has recently received renewed attention. Among these, fumarate, nitrates, sulfates and lipids (Gutierrez-Banuelos et al., 2007; Brown et al., 2011; Hristov et al., 2013) have been most studied. Leng and Preston (2010) provided a comprehensive review of the earlier literature on nitrates. Recent research with sheep (Sar et al., 2004; Nolan et al., 2010; Van Zijderveld et al., 2010) and cattle (Van Zijderveld et al., 2011; Hulshof et al., 2012) demonstrate promising results with nitrates decreasing CH_4 production by up to 50%. Because nitrate serves as a source of N to rumen microbes, the utilization of nitrates as feed additives have great potential for future field implementation (Lee and Beauchemin, 2014). Sulfates can also outcompete methanogens for H_2. In some anaerobic environments, hydrogen sulfide appears to play a role as electron donor in the reduction of nitrite to ammonia by nitrate-reducing, sulfide-oxidizing bacteria and supplementation of the diet with sulfur (Leng, 2008) or cysteine (Takahashi et al., 1997) may therefore reduce nitrite accumulation in the rumen. Sulfate is a reductant ($\Delta G^0 = -21.1$ kJ/mol of hydrogen; Ungerfeld and Kohn, 2006) and competes for electrons, reducing CH_4 production. An in vivo study with dairy cows showed significant reduction of CH_4 when sulfate was fed (16% less CH_4 than control; van Zijderveld et al., 2010).

A major pathway for propionate formation in the rumen involves the use of H_2 to reduce fumarate to propionate (Ungerfeld and Kohn, 2006). A 60% decrease was initially observed as a

result of fumarate addition to the diet (Demeyer and Hendrickx, 1967). However, the decreases observed in CH_4 production as a result of fumarate addition in seven batch culture studies were modest (mean = 6 percent; maximum = 18 percent; minimum = no change; Callaway and Martin, 1996; Asanuma et al., 1999; Lopez et al., 1999; Carro and Ranilha, 2003). In a meta-analysis of batch culture experiments it was observed that only 48 percent of added fumarate appeared to be converted to propionate and 20 percent was converted to acetate (Ungerfeld et al., 2007). In addition, another limitation for the use of fumarate or other organic acids in ruminant diets is the economic high cost of this strategy (Beauchemin et al., 2008).

The process of biohydrogenation of polyunsaturated fatty acids in the rumen draws H_2 away from methanogenesis (Czerkawski, 1986). Only 1 to 2 percent of metabolic H_2 in the rumen is used for biohydrogenation (Czerkawski and Clapperton, 1984; Jenkins et al., 2008). In addition, vegetable oils and animal fat are toxic to archaea and protozoa (Hristov et al., 2013). The greater inhibitory effect of unsaturated vs. saturated FA on rumen microbial activity reported by Palmquist and Jenkins (1980) and Nagaraja et al. (1997) does not appear to apply to CH_4 production in most studies (Beauchemin et al., 2007; Van Zijderveld et al., 2011) although a greater mitigating effect of polyunsaturated FA was observed in the analysis by Doreau et al. (2011). Persistence of the mitigating effect of dietary oil was also observed in the study of Martin et al. (2011) when flaxseed was fed to dairy cows, although it was not supported by another study from the same group using young bulls (Eugène et al., 2011). In some studies, lipids had a significant and negative impact on DMI (e.g., Martin et al., 2008), a factor that must be carefully considered both in prediction of mean responses and for risk assessment by those choosing to adopt this mitigation strategy. Some fats such as coconut oil, for example, can severely depress feed intake, fiber digestibility, and, consequently, milk production and cause

milk fat depression in dairy cows (Hristov et al., 2009, 2011; Hollmann and Beede, 2012) although they may be still beneficial as CH_4 mitigating agents (Machmüller and Kreuzer, 1999; Hristov et al., 2009). Feeding lipids to ruminants has potential to serve as a mitigation option, but more studies investigating the relationship of lipid chemical form, CH_4 production and productive parameters are needed.

Inhibition of methanogenesis by targeting archaea numbers or activity is promising, also. This should ideally be done with a concomitant stimulation of pathways that consume H_2 in order to avoid an increase in the H_2 partial pressure in the rumen and its negative effect on fermentation. Sequencing the genome of *M. ruminantium* has opened new frontiers and opportunities for inhibition of rumen methanogens and the potential to mitigate ruminant CH_4 emissions (Leahy et al., 2010). One interesting approach, using antimethanogen antibodies to suppress CH_4 production, was shown to be ineffective, in vitro (Cook et al., 2008). Compounds such as bromochloromethane, 2-bromoethane sulfonate, chloroform, and cyclodextrin have been tested, some successfully, in various ruminant species. Inhibition of methanogenesis by these compounds in vivo can be up to 60 percent with the effect of bromochloromethane shown to persist in long-term experiments (Hristov et al., 2014). However, the viability of these compounds as mitigation agents has been questioned due to concerns for animal health, food safety, or environmental impact. Bromochloromethane, for example, is an ozone-depleting agent and is banned in many countries. Among the efficacious methane inhibitors identified is 3-nitrooxypropanol. This compound was part of a developmental program designing specific small molecule inhibitors for methyl coenzyme-M (CoM) reductase, the enzyme that catalyzes the last step of methanogenesis, the reduction of methyl CoM and coenzyme-B (CoB) into methane and a CoM– CoB complex. This compound reduces CH_4 production in dairy (Haisan et al., 2013;

Reynolds et al., 2014), beef (Romero Perez et al., 2015) and sheep (Martinez-Fernandes et al., 2014) without signs of resistance. In addition, dairy cows in two studies demonstrated significant increase in body weight gain when compared with control (Haisan et al., 2014; Hristov et al., 2015).

1.5.2. Biotechnology

Biotechnological strategies are under investigation. A vaccine targeted at three methanogens decreased CH_4 production by nearly 8 percent in Australian sheep (Wright et al., 2004). However, vaccines directed towards a different set of methanogen species or tested in other geographical regions did not elicit a positive response (Wright et al., 2004). Passive immunization using antibodies produced in laying hens was tested for 3 common methanogens present in the digestive tract of animals. Treatments using whole eggs decreased transient CH_4 production in vitro but the effect was lost by the end of the 24-h incubation (Cook et al., 2008). Some bacteriocins reduce CH_4 production in vitro (Callaway et al., 1997; Lee et al., 2002). Nisin is thought to act indirectly, affecting hydrogen-producing microbes in a manner similar to that of the ionophore antibiotic, monensin (Callaway et al., 1997). A single in vivo result reported a 10 percent decrease in CH_4 emissions from sheep fed this bacteriocin (Santoso et al., 2004).

1.5.3. Genetic selection

The common assumption that CH_4 production is affected mainly by the diet has been challenged because the large variation in CH_4 emissions can be attributed to animal factors (Ellis et al., 2007; Yan et al., 2009). Between-animal variability, at the same level of performance and using similar diets, is high (Martin et al., 2010). Studies suggest that the genetic component of CH_4 production is low. However, data obtained from fattening cattle show that animals with high feed efficiency, measured as the residual feed intake, produced 20 percent less CH_4 than less

efficient animals (Nkrumah et al., 2006; Hegarty et al., 2007). Differences between these animals could be due to individual differences in rumen microorganisms associated with the rate of degradation processes and fermentation parameters and/or to intrinsic animal characteristics such as retention time of particles in the rumen, ruminal pH by differences in buffering by digesta mass, saliva production and rumen movements increasing VFA absorption. Recently, Guan et al. (2008) reported a link between the diversity of the rumen bacteria and VFA pattern with the feed efficiency in cattle. In addition, it has been shown by Pinares-Patino et al. (2003; 2007) that cows with a low retention time of particles in the rumen with similar intake produce less CH_4. Other studies have shown that the host animal controls the archaea populations in the rumen (Weimer et al., 2010; Roehe et al., 2016). Although deep metagenomic and metatranscriptomic sequencing has shown similar abundance of methanogens and methanogenesis pathway genes in high and low CH_4 emitters, the transcription of methanogenesis pathway genes was substantially increased in sheep with high CH_4 yields (Shi et al., 2014). Pinares-Patiño et al. (2013) demonstrated that there is repeatable individual variation in this trait and part of this variation is genetic, but that the heritability estimate was lower for CH_4 yield than for total daily CH_4 emissions (0.13 and 0.29, respectively). Therefore, in addition to heritability, further progress in genetic selection for low CH_4 emitters depends on better understanding of the variables involved in the observed between-animal variation of this trait. To address this problem, a recent meta-analysis using data from 40 studies concluded that variables related to physiology, such as variation in digesta retention time, can explain most of the between-animal variation in CH_4 yield, with only small variations observed in rumen fermentation variables, suggesting a minor contribution of the rumen microbiome to CH_4 production (Cabezas-Garcia et al., 2017). Herd et al. (2014) used the records of 777 young bulls for daily CH_4 production, CH_4 intensity, CH_4

yield, DMI, yearling weight and residual CH_4 production (actual minus predicted CH_4 production) and observed that CH_4 production was positively correlated with yearling weight and DMI (0.42 and 0.46, respectively), demonstrating that genetic control of CH_4 emissions in beef cattle can be performed though selective breeding. These results open an important area of study and the possibility that ruminants can be selected for low CH_4 production animals.

1.6. *Using nitrate to reduce enteric methane emissions*

Nitrates may be present in the diet of ruminants on a continuous basis, usually in the form of nitrate salts and ammonium nitrate. Nitrate uptake by plants is an essential step in the incorporation of soil N (Burrows et al., 1987), and the most common source of nitrate in the diet of ruminants are forages with cultivars from the perennial ryegrass and sorghum family (sorghum, sudan, pearl millet, and their crosses; Leng, 2008). Factors affecting nitrate concentration in forage have been well demonstrated by Leng (2008) and include 1) forage maturity, 2) soil conditions (e.g., moisture content), and 3) application of fertilizers to soil.

Nitrate is generally considered an undesirable compound in ruminant feeds because of its potential to cause intoxication (Lee and Beauchemin, 2014). However, over the last 10 years a significant number of research trials have investigated the potential of nitrate to serve as CH_4 mitigation option.

1.6.1. Effect of nitrate on enteric methane emission

The mitigation of CH_4 observed when nitrate is supplemented in the diet of ruminants is mainly related with the fate of this compound and how it affects rumen metabolism. Reduction of nitrate to NH_3 requires 4 moles of H_2, the same number of moles necessary for reduction of CO_2 into CH_4 by methanogenic archaea. Therefore, each mole of nitrate reduced to ammonia decreases CH_4 in 1 mole, or 22.4 L. The thermodynamic reaction of nitrate reduction to nitrite

(equation 5), of nitrite reduction to NH_3 (equation 6) and that of CH_4 formation (equation 7) in the rumen is as follows (Ungerfeld and Kohn 2006):

$NO_3^- + H^+ \rightarrow H_2O + NO_2^-$	(ΔG(KJ/2H)= −130 kJ/mol of hydrogen)	(Equation 6)
$NO_2^- + 3\ H_2 + 2H^+ \rightarrow NH_4^+ + 2\ H_2O$	(ΔG(KJ/2H)= −124 kJ/mol of hydrogen)	(Equation 7)
$CO_2 + 4\ H_2 \rightarrow CH4 + 2\ H_2O$	(ΔG(KJ/2H)= −16.9 kJ/mol of hydrogen)	(Equation 5)

The potential of nitrate to reduce CH_4 production without resulting in signs of toxicity has been confirmed in sheep (Nolan et al. 2010; van Zijderveld et al. 2010), dairy (van Zijderveld et al. 2011; Guyader et al., 2015) and beef cattle (Lee et al., 2015a, b) without resulting in clinical signs of toxicosis. In order to harvest the full potential of this compound both as a CH_4 mitigation tool and as a source of NPN the rumen ecosystem should be previously acclimated. Even though nitrate is commonly present in different feedstuffs fed to ruminants (Leng, 2008), specially forages, the concentrations are usually low, and if the rumen microorganisms are not adapted, nitrite may accumulate in the rumen and be absorbed into the blood leading to methemoglobinemia.

In a recent meta-analysis, Lee and Beauchemin (2014) investigated the relationship between nitrate feeding and CH_4 production (9 studies and 29 treatments) and enteric CH_4 emissions were linearly decreased with increasing levels of nitrate consumed by animals (R^2 = 0.80, $P < 0.01$). In the studies investigated by Lee and Beauchemin (2014), the dosages of nitrate ranged from 0.03 to 1.19 g of nitrate per kg^{-1} BW and in all studies nitrate significantly reduced CH_4 emissions when compared to non-nitrate treatments. Consistency on CH_4 abatement is an important feature when developing strategies to reduce CH_4 emissions by ruminants (Hristov et al., 2013). In addition, some mitigation approaches effectively reduced enteric CH_4 emissions in

the short term,but are not effective in the long term because of microbial adaptation to the compound (*e.g*., bromochloromethane). Therefore, the importance of conducting long-term *in vivo* experiments has been highlighted for the research on enteric methane emissions (Hristov et al., 2013). From this perspective, nitrate is a good candidate feed additive to mitigate enteric CH_4 emissions as it appears to be effective and persistent over time as observed in long-term experiments conducted *in vitro* (Capelari et al., 2017) and *in vivo* in dairy (van Zijderveld, 2010) and in beef (Lee et al., 2017 a,b).

1.6.2. Effect of nitrate on production parameters

It is often assumed that feeding nitrate has negative effects on animal performance because of its potential toxicity. Reduced intake, weight gain, and milk production are anticipated symptoms of cows with milk nitrate-poisoning. An extensive review by Bruning-fann and Kaneene (1993) demonstrated that feeding nitrate at levels causing methemoglobinemia (>20-30% blood methemoglobin) could lower productivity in ruminants. van Zijderveld et al. (2011) observed no negative effects on milk production and milk composition when dairy cows were fed a diet supplemented with nitrate at 2.1% of dietary DM. Nolan et al. (2010) reported that a diet supplemented with nitrate at 3.04% of dietary DM did not affect feed intake and weight gain in sheep. In beef cattle, feed intake decreased slightly for cows fed a diet supplemented with 2.2% nitrate (dietary DM) compared with a diet with urea (isonitrogenous), but it did not differ statistically (Hulshof et al. 2012). Lee et al., (2017a,b) tested the effect of encapsulated nitrate (1.25 and 2.5 % of nitrate in diet DM) in backgrounding and finishing crossbred steers (n = 108). In the backgrounding phase, the inclusion of nitrate did not affect BW, ADG and gain:feed. The follow-up study testing encapsulated nitrate (1.25 and 2.5 % of nitrate in diet DM) in 132 crossbred steers for 150 d observed a tendency to increased ADG

compared to control with an improved gain:feed for nitrate fed animals. Olijoek et al. (2015) fed lactating dairy cows with 4 levels of nitrate (0, 5.3, 13.6 and 21.1 g of nitrate/kg of diet DM) and did not observe any significant difference in milk production, DMI and milk composition.

Capelari et al. (2017) observed that nitrate can reduce the digestion of feed components depending on diet and the dose fed with a continuous culture model. Results suggest that nitrate or a reduction product temporarily present in the media can affect utilization of some chemical components of the diets (through solubilization in ruminal fluid and/or degradation by microorganisms enzymes). Maraes et al. (1988) reported suppression of in vitro digestion of diet DM when NO_2 was present, but not when nitrate was added, thereby proposing that NO_2 was the primary factor affecting digestibility in the rumen. In the same study, NO_2 was shown to reduce solubilization of structural components of the diet, such as cellulose and hemicellulose, confirmed by a reduction in the cellulolytic and xylanolytic microbial populations (Ruminococcus flavefaciens, Ruminococcus albus and Butyrivibrio fibrisolvens) with concomitant reduction in cellulase and xylanase activity. Reduction of ADF digestibility has been reported in beef heifers fed increasing levels of nitrate on a barley silage and ground corn-based diet despite an increase in total-tract DM digestibility, likely due to increased digestibility of starch (Lee et al., 2015a). Asanuma et al. (2015) reported increased starch-utilizing organisms when goats were fed 9 g/d nitrate. Encapsulation of nitrate, especially in beef diets, may limit the synchronization between readily available carbohydrates and N sources to rumen starch utilizers, therefore reducing the growth of these microorganisms. Olijoek et al. (2015) evaluated the DM, CP, ADF and NDF digestibility of lactating Danish dairy cows fed 4 different levels of nitrate and did not observe any difference in ruminal, duodenal, ileal and total tract digestibility between the 4 treatments.

In all the studies discussed, a protocol was implemented for acclimation of ruminal environment to nitrate. The acclimation period may range from 10-21 d and it may be reduced if the source of nitrate is encapsulated. However, it is worth noting that a protocol for an effective acclimation procedure has not been standardized (Lee and Beachemin, 2014). For example, Hulshof et al. (2012) and van Zijderveld et al. (2011) increased nitrate by about 0.5% in the diet every 4 or 7 d, respectively, to reach the target levels (2.2 and 2.1% nitrate of dietary DM, respectively). Phuc et al. (2009) acclimatized goats to a nitrate-based diet by feeding 0.2, 0.4, 0.8, 1.6, and then 3.3% nitrate in dietary DM weekly for 5 wk. Lee et al. (2017a) acclimated beef steers in a backgrounding diet during 21 d with diet nitrate content gradually increased using a 0.8 percent unit increase (dietary DM) every 7 d. More studies are needed for standardization of the acclimation procedure.

1.7. Using monensin to reduce enteric methane emissions

The effect of monensin on rumen fermentation and animal performance has been investigated since the mid 1970's when monensin use was approved in the USA. As an ionophore, monensin has the ability to form lipid-soluble complexes with cations and mediate their transport across lipid barrier of gram positive bacteria (Russell and Strobel, 1988). Further, ionophores also inhibits the growth of fungi (Steward et al., 1987; Steward and Richardson, 1989) and transiently inhibits the growth of ciliate protozoa (Hino, 1981; Wallace et al., 1981; Guan et al., 2006; Sylvester et al., 2009). The result is a decrease in CH_4 production (Odongo et al., 2007) resulting from an increase in propionate production to the detriment of acetate and butyrate production (Guan et al., 2006), and a protein-sparing mechanism, increasing availability of true protein (Van Nevel and Demeyer, 1977). Monensin is commonly used to improve efficiency of energy (Byers, 1980) and N utilization (Ruiz et al., 2001) in cattle. Bergen and

Bates (1984) categorized rumen fermentation alterations associated with ionophore feeding into 3 major areas: 1) increased production of propionate and reduction of CH_4, resulting in increased efficiency of energy metabolism of the rumen and/or animal, 2) reduced protein degradation and deamination of amino acids, resulting in the improvement of N metabolism in the rumen and/or animal, and 3) reduced lactic acid production and froth formation in the rumen, leading to reduction of ruminal disorders. In a recent meta-analysis of 22 controlled studies, Appuhamy et al. (2013) observed that CH_4 reduction (g/d) effect of MON ranges between 12 ± 6 in dairy cows and 14 ± 6 g/d in beef steers, with mitigation outcomes similar when adjusted for dose differences between dairy and beef studies. In addition, the effect of MON on CH_4 reduction is likely transient, as suggested by Guan et al. (2006) who observed concomitant return of CH_4 output and protozoa numbers to background levels in the initial 4 wks of supplementation.

The effect of MON on nutrient digestibility has been inconsistent in both *in vitro and in vivo* studies. Wallace et al. (1981) reported a reduction in cellulose and hemicellulose digestion when the Rusitec was dosed with 10 mg/L of MON daily. Dong et al. (1999) found a reduction in cellulose (30%) and hemicellulose (21%) digestion due to MON when the Rusitec was dosed with 20.5 µM/d of monensin. In various in vitro and in vivo studies, monensin has depressed fiber digestion (Poos et al. 1979; Mir, 1989), affected fiber digestion minimally (Faulkner et al. 1985) or not at all (Duff et al.1995), and even to increased fiber digestibility (Wedegaertner and Johnson, 1983). Potential negative effects of monensin on ruminal fiber degradation are likely related to the sensitivity of the 3 primary cellulolytic species, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*, to monensin (Chen and Wolin, 1979). Factors such as the dosage used, diet chemical composition and retention time of digesta may account for variability in results (Beauchemin et al., 2008).

1.8. Objectives and rationale

Many dietary strategies to reduce CH_4 production from ruminants have been proposed and successfully tested *in vitro*, but duo to limited experimental conditions just a few have been verified *in vivo*. Among these strategies, the use of supplementary nitrate has received special attention because of its effectiveness on reducing CH_4 in different ruminant species under different dietary conditions. Monensin has been extensively utilized as a feed additive for ruminants and its effect on methanogenesis has been well documented. Although animal responses have varied in terms of CH_4 abatement, the effect of monensin on ruminal fermentation, specially methanogenesis, is well understood and it is divergent to that of nitrate. In fact, the reduction of nitrate to ammonia and the increased in fermentation efficiency through increased propionate production in the rumen can outcompete methanogens for H_2.

The overarching objective of this project is to investigate the use of supplementary nitrate in combination with monensin as a CH_4 mitigation strategy for ruminants. Because the effects of nitrate and monensin on rumen H_2 dynamics and methanogenesis are different, we hypothesized that nitrate and monensin can additively reduce enteric CH_4 production without compromise productive parameters.

The study combines *in vitro* batch culture and semi-continuous culture systems and *in vivo* approach to better understand the response of rumen microorganisms to each additive and to quantify the effect of the combination of additives on CH_4 output and other fermentation parameters.

The specific objectives of the study are:

- Quantify the dose-dependent response of nitrate and monensin on rumen fermentation parameters, *in vitro*;

- Determine if nitrate can sustain reduced CH_4 levels in the long-term if adaptation to monensin occurs in long-term cultures of mixed rumen microorganisms;
- Determine if nitrate and monensin effect on rumen fermentation is diet-dependent;
- Quantify the effect of the combination of nitrate and monensin on enteric methane emissions, *in vivo*.

LITERATURE CITED

LITERATURE CITED

Aiello, L. C., and J. C. K. Wells. 2002. Energetics and evolution of the genus *Homo*. Annual Review of Anthropology. 31:323-338.

Appuhamy, J. A. D. R. N., A. B. Strathe, S. Jayasundara, C. Wagner-Riddle, J. Dijkstra, J. France, and E. Kebreab. 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. J. Dairy Sci. 96:5161-5173.

Asanuma, N., M. Iwamoto, and T. Hino. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms in vitro. J. Dairy Sci. 82: 780-787.

Asanuma, N., S. Yokoyama, and T. Hino. 2015. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to Selenomonas ruminantium having the ability to reduce nitrate and nitrite. Anim. Sci. J. 86: 378–384

Appuhamy, J. A. D. R. N., A. B. Strathe, S. Jayasundara, C. Wagner-Riddle, J. Dijkstra, J. France, and E. Kebreab. 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. J. Dairy Sci. 96: 5161-5173.

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

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

Beauchemin, K. A., S. M. McGinn, and H. V. Petit. 2007. Methane abatement strategies for cattle: Lipid supplementation of diets. Can. J. Anim. Sci. 87:431–440.

Beauchemin, K. A., M. Kreuzer, F. O'Mara, and T. A. McAllister. 2008. Nutritional management for enteric methane abatement: A review. Austral J. Exp. Agric. 48: 21–29.

Benchaar, C., S. Calsamiglia, A. V. Chaves, G. R. Fraser, D. Colombatto, T. A. McAllister, and K. A. Beauchemin. 2008. A review of plant-derived essential oils in ruminant nutrition and production. Anim. Feed Sci. Technol. 145: 209-228.

Bergen, W. G., and D. B. Bates. 1984. Ionophores: Their effect on production efficiency and mode of action. J. Anim. Sci. 58:1465–1483.

Brown, E. G., R. C. Anderson, G. E. Carstens, H. Gutierrez-Baňuelos, J. L. McReynolds, L. J. Slay, T. R. Callaway, and D. J. Nisbet. 2011. Effects of oral nitroethane administration on enteric methane emissions and ruminal fermentation in cattle. Anim. Feed Sci. Technol. 166–167:275–281.

Bruning-Fann, C. S., and J. B. Kaneene. 1993. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. Vet. Hum. Toxicol. 35:237–253.

Burrows, G. E., G. W. Horn, R. W. McNew, L. I. Croy, R. D. Keeton, and J. Kyle. 1987. The prophylactic effect of corn supplementation on experimental nitrate intoxication in cattle. J. Anim. Sci. 64:1682–1689.

Byers, F. M. 1980. Determining effects of monensin on energy value of corn silage diets for beef cattle by linear or semi-log methods. J. Anim. Sci. 51:158–169.

Cabezas-Garcia, E. H., S. J. Krizsan, K. J. Shingfield, and P. Huhtanen. 2017. Between-cow variation in digestion and rumen fermentation variables associated with methane production. J. Dairy Sci. 100:4409–4424.

Callaway, T. R., and S. A. Martin. 1996. Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. J. Anim. Sci. 74:1982–1989.

Callaway, T. R., A.M. Carneiro De Melo, and J.B. Russell. 1997. The effect of nisin and monensin on ruminal fermentations in vitro. Current Microbiology. 35: 90–96.

Calsamiglia S, Busquet M, Cardazo PW, Castillejos L and Ferret A 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. . J. Dairy Sci. 90: 2580–2595

Capelari, M., K. A. Johnson, B. Latack, J. Roth, and W. Powers. 2017. The effect of two additives on ruminal fermentation using a semi-continuous culture system. J. Anim. Sci. 95 (Suppl. 4): 274 (Abstr.).

Capper, J. L., R. A. Cady, and D. E. Bauman. 2009. The environmental impact of dairy production: 1944 compared with 2007. J. Anim. Sci. 87: 2160-2167.

Capper, J. L. 2011. The environmental impact of beef production in the united states: 1977 compared with 2007. J. Anim. Sci. 89: 4249-4261.

Carro, M. D., and M.J. Ranilla. 2003. Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms in vitro. Brit. J. Nutr. 90: 617-623.

Cavanaugh, A., and C. A. Curry. 2016. Domestication and the evolution of agriculture. In: J.B. Losos and R.E. Lenski, editors, How Evolution Shapes our Lives. Ed. Princeton University Press, Princeton, NJ. p. 179–191.

Chen, M., and M. J. Wolin. 1979. Effect of monensin and lasalocid- sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. 38: 72–77.

Clutton-Brock, J. 1999. A Natural History of Domesticated Mammals. 2nd ed. Cambridge University Press, Cambridge.

Cook, S. R., P. K. Maiti, A. V. Chaves, C. Benchaar, K. A. Beauchemin, and T. A. McAllister. 2008. Avian (IgY) anti-methanogen antibodies for reducing ruminal methane production: In vitro assessment of their effects. Aust. J. Exp. Agric. 48:260–264.

Czerkawski, J. W. 1986. An Introduction to Rumen Studies. Pergamon Press, Oxford, UK.

Czerkawski, J. W., and J. L. Clapperton, J. L. 1984. Fats as energy-yielding compounds in the ruminant diet. In: Fats in Animal Nutrition, editor, Wiseman, pp. 249-263. Butterworths, London, U.K.

Dansen, O., W. F. Pellikaan, W. H. Hendriks, J. Dijkstra, M. P. T. Jacobs, H. Everts, and D. A. van Doorn. 2015. Daily methane production pattern of Welsh ponies fed a roughage diet with or without a cereal mixture. J. Anim. Sci. 93:1916-1926.

Demeyer, D., and H. Henderickx (1967). Competitive inhibition of “in vitro” methane production by mixed rumen bacteria. Archives Internationals de Physiologie et de Biochimie. 75:157–159.

Diamond, J. M. 1997. Guns, Germs, and Steel: The Fates of Human Societies. 1st ed. W.W. Norton Publishing. New York, NY.

Dijkstra, J., J. M. Forbes, and J. France. 2005. Quantitative Aspects of Ruminant Digestion and Metabolism. 2nd edition. Cambridge, MA;Wallingford, Oxfordshire, UK. CABI Pub.

Dong, Y., H. D. Bae, T. A. McAllister, G. W. Mathison, and K. -J Cheng. 1999. Effects of exogenous fibrolytic enzymes, α-bromoethanesulfonate and monensin on fermentation in a rumen simulation (RUSITEC) system. Can. J. Anim. Sci. 79: 491-498.

Doreau, M., C. Martin, M. Eugène, M. Popova, and D. P. Morgavi. 2011. Leviers d’action pour réduire la production de méthane entérique par les ruminants. In: M. Doreau, R. Baumont, and J. M. Perez, editors, Gaz à effet de serre en élevage bovin: Le méthane. (In French.) INRA Prod. Anim. 24:461–474.

Duff, G. C., M. I. Galyean, and M. E. Branine. 1995. Effects of adaptation to lasalocid, monensin or a daily rotation of lasalocid and monensin on in vitro fermentation of a 90% concentrate diet. Can. J. Anim. Sci. 75:129–134.

Ellis, J. L., E. Kebreab, N. E. Odongo, B. W. Mcbride, E. K. Okine,and J. France. 2007. Prediction of methane production from dairy and beef cattle. J. Dairy Sci. 90:3456–3466.

EPA, United States Environmental Protection Agency. 2014. Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990–2012. U.S. Environmental Protection Agency, Washington, DC.

Etiope, G., T. Fridriksson, F. Italiano, W. Winiwarter, and J. Theloke. 2007. Natural emissions of methane from geothermal and volcanic sources in europe. J. Volcanol. Geotherm. Res. 165:76-86.

Eugène, M., C. Martin, M. M. Mialon, D. Krauss, G. Renand, and M. Doreau. 2011. Dietary linseed and starch supplementation decreases methane production of fattening bulls. Anim. Feed Sci. Technol. 166–167:330–337.

Faulkner, D. B., T. J. Klopfenstein, T. N. Trotter, and R. A. Britton. 1985. Monensin effects on digestibility, ruminal protein escape and microbial protein synthesis on high-fiber diets. J. Anim. Sci. 61:654–660.

Franz, R., C. R. Soliva, M. Kreuzer, J. Hummel, and M. Clauss. 2011. Methane output of rabbits (Oryctolagus cuniculus) and guinea pigs (Cavia porcellus) fed a hay-only diet: Implications for the scaling of methane production with body mass in non-ruminant mammalian herbivores. Comp. Biochem. Physiol. 158:177-181.

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

Guan, L. L., D. J. Nkrumah, J. A. Basarab, and S. S. Moore. 2008. Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. FEMS Microbiol. Lett. 288:85–91.

Gutierrez-Banuelos, H., R. C. Anderson, G. E. Carstens, L. J. Slay, N. Ramlachan, S. M. Horrocks, T. R. Callaway, T. S. Edrington, and D. J. Nisbet. 2007. Zoonotic bacterial populations, gut fermentation characteristics and methane production in feedlot steers during oral nitroethane treatment and after the feeding of an experimental chlorate product. Anaerobe 13:21–31.

Guyader, J., M. Eugène, P. Nozière, D. P. Morgavi, M. Doreau, and C. Martin. 2014. Influence of rumen protozoa on methane emission in ruminants: A meta-analysis approach. Animal. 8:1816-1825.

Guyader, J., M. Eugène, B. Meunier, M. Doreau, D. P. Morgavi, M. Silberberg, Y. Rochette, C. Gerard, C. Loncke, and C. Martin. 2015. Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. J. Anim. Sci. 93:3564–3576.

Hackmann, T. J., and J. N. Spain. 2010. Invited review: Ruminant ecology and evolution: Perspectives useful to ruminant livestock research and production. J. Dairy Sci. 93: 1320-1334.

Haisan, J., Y. Sun, K. Beauchemin, L. Guan, S. Duval, D. R. Barreda, and M. Oba. 2013. Effect of feeding 3-nitrooxypropanol on methane emissions and productivity of lactating dairy cows. Adv. Anim. Biosci. 4(2):260.

Haisan, J., Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of holstein cows in mid lactation. J. Dairy Sci. 97: 3110-9.

Hawks, J. 2016. Human evolution. In: J.B. Losos and R.E. Lenski, editors, How Evolution Shapes our Lives. Ed. Princeton University Press, Princeton, NJ. p. 31–46.

Hegarty, R. S., J. P. Goopy, R. M. Herd, and B. McCorkell. 2007. Cattle selected for lower residual feed intake have reduced daily methane production. J. Anim. Sci. 85: 1479–1486.

Herd R. M., P. F. Arthur, S. H. Bird, K. A. Donoghue, and R.S. Hegarty. 2014. Genetic Variation for Methane Traits in Beef Cattle. Proc. 10th World Cong. Genet. Appl. Livest. Prod., Vancouver, Canada.

Hollmann, M., and D. K. Beede. 2012. Comparison of effects of dietary coconut oil and animal fat blend on lactational performance of Holstein cows fed a high-starch diet. J. Dairy Sci. 95:1484–1499.

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

Hristov, A. N., M. Vander Pol, M. Agle, S. Zaman, C. Schneider, P. Ndegwa, V. K. Vaddella, K. Johnson, K. J. Shingfield, and S. K. R. Karnati. 2009. Effect of lauric acid and coconut oil on ruminal fermentation, digestion, ammonia losses from manure, and milk fatty acid composition in lactating cows. J. Dairy Sci. 92:5561–5582.

Hristov, A. N., C. Lee, T. Cassidy, M. Long, K. Heyler, B. Corl, and R. Forster. 2011. Effects of lauric and myristic acids on ruminal fermentation, production, and milk fatty acid composition in lactating dairy cows. J. Dairy Sci. 94:382–395

Hristov, A. N., J. Oh, C. Lee, R. Meinen, F. Montes, T. Ott, J. Firkins, A. Rotz, C. Dell, A. Adesogan, W. Z. Yang, J. Tricarico, E. Kebreab, G. Waghorn, J. Dijkstra, and S. Oosting. 2013. Mitigation of greenhouse gas emissions in livestock production – A review of technical options for non-CO_2 emissions. P. Gerber, B. Henderson, and H. Makkar, editors. FAO Animal Production and Health Paper No. 177. FAO, Rome, Italy.

Hristov, A. N., J. Oh, F. Giallongo, T. W. Frederick, M. T. Harper, H. L. Weeks, A. F. Branco, P. J. Moatec, M. H. Deightonc, S.O. Williamsc, M. Kindermann, and S. Duval. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proceedings of the National Academy of Sciences 112: 10663.

Hulshof, R. B. A., A. Berndt, W. J. J. Gerrits, J. Dijkstra, van Zijderveld S. M, J. R. Newbold, and H. B. Perdok. 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci. 90:2317-2323.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press Publisher. New York, NY.

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Jenkins, T. C., R. J. Wallace, P. J. Moate, and E. E. Mosley. 2008. Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J. Anim. Sci. 86: 397-412.

Knapp J. R., G. L. Laur, P. A. Vadas, W. P. Weiss, and J. M. Tricarico. 2014. Invited review: Enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97:3231-3261.

Kunkel, D., R. J. Basseri, M. D. Makhani, K. Chong, C. Chang, and M. Pimentel. 2011. Methane on Breath Testing Is Associated with Constipation: A Systematic Review and Meta-analysis. Dig. Dis. Sci. 56:1612-1618.

Leahy, S. C., W. J. Kelly, E. Altermann, R. S. Ronimus, C. J. Yeoman, D. M. Pacheco, D. Li, Z. Kong, S. McTavish, C. Sang, S. C. Lambie, P. H. Janssen, D. Dey, and G. T. Attwood. 2010. The genome sequence of the rumen Methanogen Methanobrevibacter reveals new possibilities for controlling ruminant methane emissions. PLoS ONE 5:e8926.

Lee S. S., J.T. Hsu, H. C. Mantovani, and J. B. Russell. 2002. The effect of bovicin hc5, a bacteriocin from Streptococcus bovis hc5, on ruminal methane production in vitro. FEMS Microbiology Letters. 217:51–55.

Lee, C., and K. A. Beauchemin. 2014. A review of feeding supplementary nitrate to ruminant animals: Nitrate toxicity, methane emissions, and production performance. Can. J. Anim. Sci. 94: 557–570.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015a. Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers. J. Anim. Sci. 93:2391–2404.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015b. Effects of feed consumption rate of beef cattle offered a diet supplemented with nitrate ad libitum or restrictively on potential toxicity of nitrate. J. Anim. Sci. 93:4956–4965.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017a. Effects of encapsulated nitrate on growth performance, nitrate toxicity, and enteric methane emissions in beef steers: Backgrounding phase. J. Anim. Sci. 95:3700–3711.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017b. Effects of encapsulated nitrate on growth performance, carcass characteristics, nitrate residues in tissues, and enteric methane emissions in beef steers: Finishing phase. J. Anim. Sci. 95:3712–3724.

Leng, R. A. 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A report to the department of climate change. Commonwealth Government of Australia, Canberra. www.penambulbooks.com/Downloads/Leng-Final%20 Modified%20%2017-9-2008.pdf (Accessed 14 May 2017).

Leng, R. A. and T. R. Preston. 2010. Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. Livestock

Research for Rural Development; Volume 22, article no. 221. http://www.lrrd.org/lrrd22/12/leng22221.htm. (Accessed January 26, 2017.)

Li, W., W. Powers, and G. M. Hill. 2011. Feeding distillers dried grains with solubles and organic trace mineral sources to swine and the resulting effect on gaseous emissions. J. Anim. Sci. 89:3286-99.

López, S., C. Valdés, C.J. Newbold & R.J. Wallace. 1999. Influence of sodium fumarate on rumen fermentation in vitro. Brit. J. Nutr. 81: 59-64.

Lyimo T. J., A. Pol and J. M. Huub. 2002. Sulfate reduction and methanogenesis in sediments of mtoni mangrove forest, Tanzania. AMBIO. 31:614-616.

Madigan M. T., J. M. Martinko, and T. D. Brock. 2006. Brock Biology of Microorganisms. 11th ed. Upper Saddle River, NJ: Pearson Prentice Hall.

Lovett D. K., S. Lovell, l. Stack, J. Callan, M. Finlay, J. Conolly, and F.P. O'Mara. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest. Prod. Sci. 84:135–146.

Machmüller, A., and M. Kreuzer. 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79:65–72.

McAllister, T. A., and C. J. Newbold. 2008. Redirecting rumen fermentation to reduce methanogenesis. Austral J. Exp. Agric. 48:7–13.

Martin, C., H. Dubroeucq, D. Micol, J. Agabriel, and M. Doreau. 2007. Methane output from beef cattle fed different high-concentrate diets. In Proceedings of the British Society of Animal Science, 2–4 April 2007, Southport, UK, p. 46.

Martin, C., J. Rouel, J. P. Jouany, M. Doreau, and Y. Chilliard. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. J. Anim. Sci. 86:2642–2650.

Martin C., D. P. Morgavi, M. Doreau. 2010. Methane mitigation in ruminants: From microbe to the farm scale. Animal. 4:351-365.

Martin, C., D. Pomiès, A. Ferlay, Y. Rochette, B. Martin, Y. Chilliard, D. P. Morgavi, and M. Doreau. 2011. Methane output and rumen microbiota in dairy cows in response to longterm supplementation with linseed or rapeseed of grass silage- or pasture-based diets. Proc. N.Z. Soc. Anim. Prod. 71:242–247.

Martínez-Fernández, G., L. Abecia, A. Arco, G. Cantalapiedra-Hijar, A. I. Martín-García, E. Molina-Alcaide, M. Kindermann, S. Duval, and D. R. Yáñez-Ruiz. 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. Journal of Dairy Science 97: 3790-3799.

Milton, K. 1999. A hypothesis to explain the role of meat-eating in human evolution. Evol. Anthropol. 8:11-21.

Ministério da Ciência e Tecnologia. 2002. Segundo inventário brasileiro de emissões e remocoes antrópicas de gases de efeito estufa: Emissões de metano da pecuária. Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) – Ministério da Ciência e Tecnologia. (https://www.alice.cnptia.embrapa.br/bitstream/doc/921485/1/2011MZ02.pdf).

Mir, Z. 1989. Monensin, chlortetracycline and tylosin effects on performance and digestion in lambs fed a ground alfalfa diet. Can. J. Anim. Sci. 69:505–508.

Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4: 1024-1036.

Nagaraja, T. G., C. J. Newbold, C. J. Van Nevel, and D. I. Demeyer. 1997. Manipulation of ruminal fermentation. In: P. N. Hobson and C.S. Stewart, editors, The Rumen Microbial Ecosystem. 2nd. Ed. Blackie Academic & Professional, London, UK. p. 523–632.

Nkrumah, J. D., E. K. Okine, G. W. Mathison, K. Schmid, C. Li, J. A. Basarab, M. A. Price, Z. Wang, and S. S. Moore. 2006. Relationships of feedlot feed efficiency, performance and feeding behaviour with metabolic rate, methane production, and energy partitioning in beef cattle. J. Anim. Sci. 84:145–153.

Moss, A. R, J. P. Jouany, and J. Newbold. 2000. Methane production by ruminants: its contribution to global warming. Annales de Zootechnie. 49:231–253.

Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. Anim. Prod. Sci. 50:801–806.

OECD/FAO. 2017. OECD-FAO Agricultural Outlook 2017-2026, OECD Publishing, Paris, France. http://dx.doi.org/10.1787/agr_outlook-2017-en (accessed November 16, 2017).

Olijhoek, D. W., A. L. F. Hellwing, M. Brask, M. R. Weisbjerg, O. Højberg, M. K. Larsen, J. Dijkstra, E. J. Erlandsen, and P. Lund. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. J. Dairy Sci. 99:6191–6205.

Odongo, N. E., R. Bagg, G. Vessie, P. Dick, M. M. Or-Rashid, S. E. Hook, J. T. Gray, E. Kebreab, J. France, and B. W. McBride. 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. J. Dairy Sci. 90:1781–1788.

Palmquist, D. L., and T.C. Jenkins. 1980. Fat in lactation rations: review. J. Dairy Sci. 63: 1–14.

Phuc, H. T., D. H, Quang, T. R. Preston, and R. A. Leng. 2009. Nitrate as a fermentable nitrogen supplement for goats fed forage based diets low in true protein. Livestock Research for Rural Development. 21: 1-14.

Pinares-Patiño, C. S., M. J. Ulyatt, K. R. Lassey, T. N. Barry, and C. W. Holmes. 2003. Rumen function and digestion parameters associated with differences between sheep in methane emissions when fed chaffed Lucerne hay. J. Agric. Sci. 140:205–214.

Pinares-Patino, C. S., G. C. Waghorn, A. Machmuller, B. Vlaming, G. Molano, A. Cavanagh, and H. Clark. 2007. Methane emissions and digestive physiology of non-lactating dairy cows fed pasture forage. Canadian J. Anim. Sci. 87:601–613.

Pinares-Patiño, C. S., S. M. Hickey, E. A. Young, K. G. Dodds, S. Maclean, G. Molano, E. Sandoval, H. Kjestrup, R. Harland, C. Hunt, N. K. Pickering, and J. C. McEwan. 2013. Heritability estimates of methane emissions from sheep. Animal 7:316–321.

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

Powers, W., B. Auvermann, N. A. Cole, C. Gooch, R. Grant, J. Hatfield, P. Hunt, K. Johnson, A. Leytem, W. Liao, and J. M. Powell. 2014. Quantifying greenhouse gas sources and sinks in animal production systems. In: M. Eve, D. Pape, M. Flugge, R. Steele, D. Man, M. Riley-Gilbert, and S. Biggar, editors, Quantifying Greenhouse Gas Fluxes in Agriculture and Forestry: Methods for Entity-Scale Inventory. Technical Bulletin 1939. chap 5. Office of the Chief Economist, U.S. Department of Agriculture, Washington, DC.

Reynolds, C. K., D. J. Humphries, P. Kirton, M. Kinderman, S. Duval, and W. Steinberg. 2013. Effect of incremental doses of 3-nitrooxypropanol on methane production, digestion, and rumen parameters in lactating dairy cows. Adv. Anim. Biosci. 4(2):261.

Robertson, L. J., and G. C. Waghorn. 2002. Dairy industry perspectives on methane emissions and production from cattle fed pasture or total mixed rations in New Zealand. Proc. New Zealand Soc. Anim. Prod. 62: 213–218.

Romero-Perez, A., E. K. Okine, S. M. McGinn, L. L. Guan, M. Oba, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2015. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. Journal of Animal Science 93: 1780-1791.

Roehe, R., R. J. Dewhurst, C.-A. Duthie, J. A. Rooke, N. McKain, D. W. Ross, J. J. Hyslop, A. Waterhouse, T. C. Freeman, M. Watson, and R. J. Wallace. 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomics gene abundance. PLoS Genet.12:e1005846.

Ruiz, R., G. L. Albrecht, L. O. Tedeschi, G. Jarvis, J. B. Russell, and D. G. Fox. 2001. Effect of monensin on the performance and nitrogen utilization of lactating dairy cows consuming fresh forage. J. Dairy Sci. 84:1717–1727.

Rosegrant, M. W., M. Agcaoili-Sombilla, and N. D. Perez. 1995. Global food projections to 2020: Implications for investment. International Food Policy Research Institute, Washington D.C.

Russell, J. B., and S. A. Martin. 1984. Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms in vitro. J. Anim. Sci. 59:1329–1338.

Russell, J. B., and H. J. Strobel. 1988. Effect of additives on in vitro ruminal fermentation: a comparison of monensin and bacitracin, another gram-positive antibiotic. J. Anim. Sci. 66:552-8.

Santoso, B., B. Mwenya, C. Sar, Y. Gamo, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, and J. Takahashi. 2004. Effects of supplementing galactooligosaccharides, Yucca schidigera or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livestock Production Science. 91: 209–217.

Sar, C., B. Santoso, B. Mwenya, Y. Gamo, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, and J. Takahashi. 2004. Manipulation of rumen methanogenesis by the combination of nitrate with β 1-4 galacto-oligosaccharides or nisin in sheep. Anim. Feed Sci. Technol. 115:129–142.

Sauvant, D., and S. Giger-Reverdin. 2007. Empirical modelling meta-analysis of digestive interactions and CH_4 production in ruminants. In: I. Ortigues-Marty, N. Miraux and W. Brand- Williams, editors, Energy and Protein Metabolism and Nutrition. EAAP publication no. 124. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 561.

Steinfeld, H., P. Gerber, T. Wassenaar, V. Castel, M. Rosales, and C. De Haan. 2006. Livestock’s long shadow. Food and Agriculture Organization of the United Nations, Rome, Italy.

Stewart. C. S., C. A. McPherson, and E. Cansunar. 1987. The effect of lasalocid on glucose uptake, hydrogen production and the solubilization of straw by the anaerobic fungus Neocallimastix frontalis. Lett. Appl. Microbio. 5: 5-7.

Stewart, C. S., and A. J. Richardson. 1989. Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. Appl. Bacteriol. 66:85-93.

Sylvester, J. T., S. K. R. Karnati, B. A. Dehority, M. Morrison, G. L. Smith, N. R. St-Pierre, and J. L. Firkins. 2009. Rumen protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci. 92:256–269.

Takahashi, J., A. S. Chaudhry, R. G. Beneke, Suhubdy, and B. A. Young. 1997. Modification of methane emission in sheep by cysteine and a microbial preparation. Sci. Total Environ. 204:117–123.

Thorpe, A. 2009. Enteric fermentation and ruminant eructation: the role (and control?) of methane in the climate change debate. Climate Change. 93: 4017-431.

Tubiello, F. N., M. Salvatore, A. F. Ferrara, J. House, S. Federici, S. Rossi, and R. Biancalani. 2015. The contribution of agriculture, forestry and other land use activities to global warming, 1990–2012. Global Change Biology. 21: 2655-2660.

Ungerfeld, E. M. and R. A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 55–85.

Ungerfeld, E. M., R. A. Kohn, R. J. Wallace, and C. J. Newbold. 2007. A meta-analysis of fumarate effects on methane production in ruminal batch cultures. J. Anim. Sci. 85: 2556–2561.

VandeHaar, M. J., and N. St-Pierre. 2006. Major advances in nutrition: Relevance to the sustainability of the dairy industry. J. Dairy Sci. 89:1280–91.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. 2 ed. Cornell University Press, Ithaca, NY.

Van Nevel, C. J. and D. I. Demeyer. 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. 34:251–257.

Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93:5856–5866.

van Zijderveld, S. M. 2011. Dietary strategies to reduce methane emissions from ruminants. PhD Thesis. Wageningen Univ., Wageningen, the Netherlands.

Van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94: 4028–4038.

Zeder, M. A., and B. Hesse. 2000. The initial domestication of goats (capra hircus) in the Zagros

mountains 10,000 years ago. Science 287: 2254-2257.

Yan, T., M. G. Porter, and C. S. Mayne. 2009. Prediction of methane emission from beef cattle using data measured in indirect open circuit respiration calorimeters. Animal 3:1455–1462.

Yoshida H., H. Tokumoto, R. Ishii, and K. Ishii. 2009. Efficient, high-speed methane fermentation for sewage sludge using subcritical water hydrolysis as pretreatment. Bioresour. Technol. 100:2933-2939.

Wallace, R. J., J. W. Czerkawski, and G. Breckenridge. 1981. Effect of monensin on the fermentation of basal rations in the rumen simulation technique (Rusitec). Br. J. Nutr. 46:131–148.

Wedegaertner, T. C., and D. E. Johnson. 1983. Monensin effect on digestibility, methanogenesis and heat increment of cracked corn-silage diet fed to steers. J. Anim. Sci. 57: 168–177.

Weimer, P. J., D. M. Stevenson, H. C. Mantovani, and S. L. C. Man. 2010. Host specificity of the ruminal bacterial community of the dairy cow following near-total exchange of ruminal contents. J. Dairy Sci. 93:5902–5912.

White, R. R., and M. B. Hall. 2017. Nutritional and greenhouse gas impacts of removing animals from US agriculture. Proc. Natl. Acad. Sci. U.S.A. 114: E10301.

Wood B., and M. Collard. 1999. The human genus. Science 284:65-71

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

Wuebbles, D. J., D. W. Fahey, K. A. Hibbard, B. DeAngelo, S. Doherty, K. Hayhoe, R. Horton, J. P. Kossin, P. C. Taylor, A. M. Waple, and C. P. Weaver. 2017. Executive summary. In: Wuebbles, D. J., D. W. Fahey, K. A. Hibbard, D. J. Dokken, B. C. Stewart, and T. K. Maycock, editors, Climate Science Special Report: Fourth National Climate Assessment, Volume I. U.S. Global Change Research Program, Washington, DC. p. 12-34.

CHAPTER 2. THE EFFECT OF NITRATE AND MONENSIN ON *IN VITRO* RUMINAL FERMENTATION

The effect of nitrate and monensin on *in vitro* ruminal fermentation[1]

M. Capelari* and W. Powers§ 2

*Department of Animal Science, Michigan State University, East Lansing, 48824 and

§ University of California, Oakland, 94607;

[1]The authors thank CAPES foundation, Ministry of Education of Brazil, for providing funds through the scholarship number 13634/2013-5 for the Ph.D program of the first author.

[2]Corresponding author: wendy.powers@ucop.edu

2.1. Abstract

Two experiments (**EXP1** and **EXP2**) evaluated the effect of calcium ammonium nitrate decahydrate (**NIT**) and sodium monensin (**MON**) on *in vitro* fermentation parameters of 2 contrasting diets (100:0 and 10:90 forage to concentrate ratio, respectively). Diet addition of NIT (0, 1.25 and 2.5 g/100g DM) and MON (0, 3 and 6 mg/L) were tested alone and combined (9 treatments total; 5 bottles per treatment). Mixed ruminal microorganisms were incubated in anaerobic media containing 0.5 g of substrate diet, one of 9 treatments and 40 mL buffer solution. Incubations were carried out in batch cultures for 48 h at 39°C. Headspace gas volume was measured and sampled at 4, 8, 12, 24 and 48 h and VFA profile was assessed at 48 h. Total gas production was reduced by NIT (87.9 vs. 94.6 mL; $P < 0.01$) and MON (78.6 vs. 94.6 mL; $P < 0.01$) and further reduced only in EXP2 when additives were combined (161.1 vs. 196.9 mL; $P < 0.01$). Methane production decreased when NIT and MON were added in EXP 1 (3.4 and 4.1 vs. 9.1 mL; $P < 0.01$) and EXP 2 (8.3 and 7.6 vs. 15.3 mL; $P < 0.01$), and further reduced when the additives were combined in EXP1 and EXP 2 (1.1 and 1.5 vs. 9.1 and 15.3 mL, respectively; $P < 0.01$). Both EXP demonstrated a significant increase in nitrous oxide (**N_2O**; $P < 0.01$) when NIT was added. The IVDMD was reduced only when the additives were combined at higher doses in EXP1 and EXP2 (31.7 vs. 37.5 and 76.6 vs. 79.9 %, respectively; $P < 0.01$). Net VFA production was not affected by treatments ($P > 0.10$), but molar proportions of acetate and butyrate were reduced by MON ($P < 0.01$). Propionate molar proportion was increased in both experiments by MON ($P < 0.01$) and further increased in EXP2 when additives were combined at lower doses ($P < 0.01$). The acetate:propionate (**A:P**) ratio was reduced in EXP1 and EXP2 by

MON (1.2 vs. 2.8 and 1.0 vs. 2.3, respectively; $P < 0.01$) and further reduced in EXP2 by the combination of additives (0.8 vs. 2.3; $P < 0.01$). Fermentation efficiency (%) was increased by MON (81.7 vs. 73.7 %, $P < 0.01$) and further increased in EXP2 when additives were combined at lower doses (87.2 vs. 76.6 %; $P < 0.01$). The combination of NIT and MON in two contrasting diets proved beneficial by altering fermentation products towards lower CH_4 and more propionate, however the addition of NIT consistently increased N_2O production. Negative effects of additives to IVDMD were only found when additives were combined at higher doses.
Key words: in vitro, methane, monensin, nitrate, nitrous oxide

2.2. Introduction

Methane (**CH_4**) production is an inherent consequence of microbial degradation of dietary carbohydrates in the foregut of ruminant animals that allows continued microbial activity (Hungate, 1966). However, CH_4 is a potent greenhouse gas (Gerber et al., 2015) and a dietary energy loss for the host animal (Blaxter, 1966). As a greenhouse gas, the largest biogenic source of CH_4 is enteric fermentation from ruminants (US EPA, 2006), while 2 – 12 % of dietary GE is lost in the form of CH_4 (Johnson and Johnson, 1995). Reducing CH_4 production from ruminants will contribute to global efforts to control global warming while offering potential improvements for animal energy metabolism.

Rumen CH_4 formation is dependent upon carbon dioxide (**CO_2**) and hydrogen (**H_2**) availability (Miller, 1995), and the metabolic pathways involved in H_2 production and utilization are important factors that should be considered when developing strategies to reduce CH_4 emissions from ruminants (Martin et al., 2010). In the rumen, nitrate (**NIT**) is reduced to ammonia (**NH_3**), and this reaction provides an alternative, and energetically more favorable, pathway for disposal of H_2 generated during fermentation (Ungerfeld and Kohn, 2006).

Stoichiometrically, if all NIT is reduced to NH_3, then CH_4 production should decrease by 25.8 g/100 g NIT (Leng and Preston, 2010). Monensin (**MON**) reduces acetate-producing bacteria and protozoal populations, especially *Entodinium spp*, that utilizes CO_2 for H_2 disposal (Chen and Wolin, 1979; Guan et al., 2006), consequently reducing CH_4 production.

Because NIT and MON offer different mechanisms to control methanogenesis, we hypothesized that combining additives would further reduce CH_4 production when compared to additives alone with no impact to production parameters. The objective of this study was to assess the effect of a combination of NIT and MON, *in vitro*, on rumen fermentation parameters.

2.3. Materials and Methods

Experiments were conducted at Michigan State University and consisted of 2 independent rumen batch culture trials. The 2 experiments (**EXP1** and **EXP2**) were independently performed to assess the dose and combination effect of NIT and MON on rumen fermentation parameters (gas production, CH_4, N_2O, VFA, pH and IVDMD) using 2 contrasting diet substrates. All animal procedures were approved and followed internal guidelines recommended by the Animal Care and Use Committee of Michigan State University.

2.3.1. Experimental design and treatments

For each experiment, 3 different doses of calcium ammonium nitrate decahydrate (NIT; $5Ca(NO_3)_2.NH_4NO_3.10H_2O$; Yara International, Oslo, Norway; 0, 1.25, 2.5g/100g DM) and monensin sodium (MON; $C_{36}H_{61}NaO_1$; Sigma Aldrich Corporation, St. Louis, USA; 0, 3, 6 mg/L of buffered rumen fluid) were tested independently and in combination, resulting in 9 treatments. Serum bottles (165 mL volume) were used to simulate the rumen *in vitro* and each bottle was considered an experimental unit. Each of the 9 treatments had 5 replicates, totaling 45 bottles. Six extra bottles served as blanks for VFA (3 bottles) and IVDMD (3 bottles). The VFA blanks

contained the diet substrate (0.5 g) and buffered rumen fluid (10 and 40 mL of processed rumen fluid and buffer solution, respectively) and bottles were immersed in ice-water bath for 15 min to cease fermentation immediately following addition of buffered rumen fluid. The average VFA concentration from blanks was then used to calculate net VFA production at the end of 48-h incubation (Net VFA production = final VFA accumulation at 48 h – blanks average).

Two stock solutions of MON were prepared 1 d before the experiments by dissolving either 15 or 30 mg of MON in 10 mL of 100% ethanol. Immediately before the serum bottles were sealed, 0.1 mL of the stock solution was transferred to the respective bottle already containing 50 mL of buffered rumen fluid, producing 3 and 6 mg of MON per L of buffered rumen fluid. An equal amount of pure ethanol was added to all remaining bottles to equalize the amount of ethanol added to all treatments. The NIT source was directly mixed in the diets before incubation.

The basal diet used in EXP1 consisted of 100% ground grass hay while in EXP2 the basal diet consisted of 90% ground corn and 10% ground grass hay (Table 1). Because the NIT source contained 17.6% N and 19.6% Ca, experimental diets were balanced with addition of urea and limestone to maintain the same levels of N and Ca across treatments. The basal diet was ground in a Wiley (Thomas Scientific, Swedesboro, NJ) mill to pass a 1 mm screen and 0.5 g of the processed diet was added to all bottles.

2.3.2. Incubation conditions and rumen inoculum source

Inoculum donors were housed at the Michigan State University Dairy Cattle Teaching and Research Facility. Rumen inoculum for EXP1 was collected from 2 ruminally fistulated dry cows consuming mixed grass hay only, while inoculum for EXP2 was collected from 3 ruminally fistulated lactating cows fed a 50:50 forage:concentrate (DM basis - approximately

18% ground corn, 18% alfalfa haylage, 15% soybean meal, 15% high moisture corn and 18% corn silage) in the form of total mixed ration (**TMR**). Diets of rumen fluid donors did not contain any ionophore or supplementary nitrate. Rumen fluid was collected 3 h following the morning feeding from 5 different rumen locations of each donor then transferred to a pre-heated 10-L thermos. The rumen inoculum was immediately transported to the laboratory where it was blended and filtered through 2 layers of cheesecloth.

The processed fluid (10 mL) was transferred under continuous CO_2 flow to pre-warmed serum bottles containing 40 mL of buffered artificial saliva (pH – 8.1; buffered rumen fluid pH not measure at 0 h; 35 g/L $NaHCO_3$, 4 g/L $(NH_4)HCO_3$, 5.7 g/L Na_2HPO_4, 6.2 g/L KH_2PO_4, 0.6 g/L $MgSO_4.7H_2O$, 13.2 g/L $CaCl_2.2H_2O$, 10 g/L $MnCl_2.4H_2O$, 1 g/L $CoCl_2.6H_2O$ and 8.0 g/L $FeCl_3$-$6H_2O$; Goering and Van Soest, 1970), fitted with a rubber stopper and crimped-sealed with aluminum caps. Before incubation (0 h) any additional pressure was released from bottles by inserting a 16-gauge needle through the rubber septa. Bottles were incubated at 39°C (±0.3) in an incubator (Ambi-hi-low chamber, Lab Line Instruments Inc, Melrose Park, IL) and hand-shaken every 2 h.

2.3.3. Sampling and measurements procedures

Gas pressure in head space was measured with a relative pressure gauge (Model Media Gauge, SSI Technologies, Jonesville, WI) at 4, 8, 12, 24 and 48 h of incubation and used for calculation of total gas produced during each interval (Eq. 1). At the same time points a 5-mL sample of gas was collected from each bottle using a precise syringe (Hamilton Company, Reno, NV) and immediately transferred to 20-mL vials containing 15 mL of ultra-high purity N_2 gas. Following headspace sampling, additional accumulated gas was released from the system by inserting a 16-gauge needle through rubber septa of all serum bottles. Methane concentrations

were obtained by gas chromatography equipped with a flame ionization detector (FID; Shimadzu 2014; Shimadzu, Addison, IL) and nitrous oxide (N_2O) concentrations were obtained using an electron capture detector (ECD; Shimadzu 2014; Shimadzu, Addison, IL). Carrier gas was ultra-purity N_2 gas, with total flow of 40 mL/min and purge flow of 0.5 mL/min. The column oven was maintained at 75°C, FID at 250°C and ECD at 325°C. The GC was equipped with an automatic headspace sampler (COMBI Pal LEAP Technologies, Carrboro, NC).

Gas production at each time point was calculated as follows:

Eq. 1:

$$V_{GAS} = \frac{V_{BT} - V_R}{P_{EL}} \times P_{BT}$$

where Vgas is the volume of gas production at each time point, mL; V_{BT} is the volume of serum bottle, 165 mL; V_R is the volume of buffered rumen fluid, 50 mL; P_{EL} is the atmospheric pressure in East Lansing, MI adjusted for temperature and humidity, 14.69 (psi); and P_{BT} are the pressure measurements from the gauge, psi. Total gas production, CH_4 and N_2O production at each time point (4, 8, 12, 24 and 48 h) were added to obtain total gas production over 48-h incubation.

After the last gas sampling at 48 h, fermentation was stopped by placing serum bottles in ice-water bath for 15 min. Bottles were then opened and pH was measured with a pH meter (Model HQ40d Portable pH meter, BACH Co, Reno, NV) by submerging the probe approximately 3 cm in the fermentation media. Fermentation media (7 mL) was then collected and transferred to 15-mL plastic centrifuge tubes. The tubes were initially centrifuged with 15,000 × g for 15 min at 4 °C. Five mL of the supernatant was transferred to a second set of 15-mL tubes and 1 mL of 25% m-phosphoric acid was added. After 15 min, samples were recentrifuged under the same conditions and 1 mL of supernatant was collected and transferred

to GC vials for VFA analysis. Sample VFA profile was obtained by GC equipped with FID (Shimadzu 2010; Shimadzu, Addison, IL). Carrier gas was ultra-high purity He, flow rate was set at 62 mL /min and purge flow was 3 mL /min. Oven temperature ramp was set to 150 °C for 2 min with further increments of 15 °C/min until reach 220 °C.

Samples of ground corn, mixed grass hay and ingredients used in the TMR fed to donor cows of EXP2 were analyzed for DM, CP, NDF, non-fiber carbohydrates, ether extract, Ca and P by wet chemistry (Dairy One, Forage Analysis Laboratory, Ithaca, NY).

2.3.4. Fermentation balance calculations

All equations used here are described in detail by Wolin (1960), Demeyer and Tamminga (1987) and Demeyer (1991). In summary, the H_2 recovery (%; Eq. 4) was calculated from the ratio of H_2 utilized and H_2 produced (μ moles/ mL; Eq. 2 and 3, respectively). The H_2 produced as fermentation end products and H_2 consumed to form CH_4 and VFA were determined from molar concentration of acetate (C_2), propionate (C_3), butyrate (C_4), isovalerate (Ci_5), valerate (C_5) and CH_4. The equations do not account for H_2 released in the gaseous form, lactate, microbial mass, and potential acetate produced via reductive acetogenesis, and were calculated as follows:

$$H_2 \text{ utilized} = (2 \times C_3) + (2 \times C_4) + (4 \times CH_4) + Ci_5, \quad [2]$$

in which H_2 utilized is expressed as micromoles per milliliters,

$$H_2 \text{ produced} = (2 \times C_2) + C_3 + (4 \times C_4) + (2 \times C_5) + (2 \times Ci_5), \quad [3]$$

in which H_2 produced is expressed as micromoles per milliliters,

$$H_2 \text{ recovery} = (H_2 \text{ utilized}/ H_2 \text{ produced}) \times 100, \quad [4]$$

in which H_2 recovery is expressed as micromoles per milliliters,

The amount of hexose (C_6) fermented was calculated as follows:

$$\text{hexose fermented} = (0.5 \times C_2) + (0.5 \times C_3) + C_4 + C_5, \quad [5]$$

in which hexose fermented is expressed in micromoles per milliliter.

Fermentation efficiency was calculated by considering the heat of combustion of glucose, acetate, propionate and butyrate and their molar concentration (mM), as follows:

$$\text{fermentation efficiency} = \{[0.62 \times C_2) + (1.09 \times C_3) + (0.78 \times C_4)]/(C_2 + C_3 + C_4)\} \times 100, \quad [6]$$

in which fermentation efficiency is expressed as a percent.

2.3.5. IVDMD

Three extra bottles per treatment were processed and incubated as described previously in order to determine IVDMD. The bottles were fitted with a 16-gauge needle for gas release and at the end of 48-h incubation, fermentation media with undegraded substrate was filtered using the same apparatus and procedure described by Goering and Van Soest (1970). Hot water (20 mL; 90-100 °C) was used to clean all serum bottles and avoid unaccounted undegraded material. The substrate DM content was determined by drying at 55 °C in a forced-air oven for 48 h followed by weighing at 105° C, and the fraction of IVDMD for each bottle was calculated by subtracting the dry residue weight (corrected for the blank) from the dry substrate weight and dividing by the dry weight of substrate.

2.3.6. Statistical analysis

All statistical analyses used the mixed procedure of SAS (SAS Inst., Inc., Cary, NC), using bottle as the experimental unit. Means for all variables were obtained by LSMEANS. The model was as follows:

$$Y_{ij} = \mu + \beta_i + T_j + e_{ij}$$

Where μ is the overall mean, β is the fixed effect of the *I*th bottle (β = 45 for gas variables, pH, VFA and fermentation balance parameters and 27 for IVDMD), T is the fixed effect of jth treatment (T= 1 to 9) and e is the residual term ($e_{ij} \sim N(0, \Sigma)$). Means were separated by Tukey and a level of 0.05 was used to determine significance, with tendencies associated with P-values between 0.05 and 0.10.

2.4. Results

2.4.1. EXP1 (100:0 roughage:concentrate)

The addition of NIT and MON reduced total gas production over 48-h incubation when compared to control (87.9 and 78.6 vs. 94.6 mL, respectively; $P < 0.01$; Table 2). When compared to control, NIT and MON significantly reduced CH_4 (mL, mL/g DM and mL/g DMD; $P < 0.01$) production. The combination of NIT + MON further reduced CH_4 when compared to control (1.1 vs. 9.1 mL; $P < 0.01$). A significant increase in N_2O production was observed when NIT was added ($P < 0.01$). There was no effect of NIT and MON on IVDMD compared to control ($P > 0.10$), however the combination of both additives at higher doses reduced IVDMD when compared to control (31.7 vs. 37.5 %; $P < 0.01$). Treatments had no effect ($P > 0.10$) on pH levels.

Total VFA production tended to decrease due to MON (46.8 vs. 48.5 mM; P = 0.08; Table 3). Molar proportion of acetate and butyrate were reduced by MON ($P < 0.01$) while propionate was increased ($P < 0.01$). The A:P ratio was reduced by MON when compared to control (1.2 vs.

2.8; P < 0.01) and tended to increase due to NIT (*P* = 0.06). Isobutyrate and isovalerate were not affected by treatments in EXP1 (*P* > 0.10), but valerate was reduced by MON (1.7 vs. 2.5 mol/ 100 mol; P < 0.01) and increased by NIT (2.9 vs. 2.5 mol/ 100 mol; *P* < 0.05).

Metabolic H_2 balance, especially H_2 recovery, was affected by both NIT and MON and reflected mainly the decrease in CH_4 output by NIT and the change in A:P ratio caused by MON addition. Total recovered H_2 was significantly reduced by NIT when compared to control (39.9 vs. 53.5%; *P* < 0.01; Table 3) and increased by MON (66.4 vs. 53.5%; *P* < 0.01) and the combination of additives (59.2 vs. 53.5%; *P* < 0.01). No effect of treatments was found on total hexose fermented (*P* > 0.10), but fermentation efficiency was increased by MON when compared to control (78.8 vs. 70.9 %, respectively; *P* < 0.01).

2.4.2. EXP2 (10:90 roughage:concentrate)

Total gas production (mL) when compared to control was reduced by NIT (181.7 vs. 196.9 mL; *P* < 0.01), MON (168.8 vs. 196.9 mL; *P* < 0.01) and further reduced by NIT + MON (161.2 vs. 196.9; *P* < 0.01; Table 4). when compared to control, CH_4 (mL, mL/g DM and mL/g DMD) was decreased by NIT (8.3 vs. 15.3 mL; *P* < 0.01), MON (7.6 vs. 15.3 mL; *P* < 0.01) and further reduced when additives were combined (1.5 vs. 15.3 mL; *P* < 0.01). The addition of NIT increased N_2O production (*P* < 0.01) and the combination of NIT + MON further increased N_2O production (*P* < 0.01). The IVDMD, similarly to EXP1, was not affected by NIT or MON (*P* > 0.10), but was reduced when additives were combined at higher doses (76.7 vs. 79.9 %; *P* < 0.01). No treatment effect was detected on pH levels (*P* > 0.10).

Total VFA production tended to decrease by NIT addition (*P* = 0.07), but no effect was found for MON or NIT + MON (Table 5). The molar proportion of acetate and butyrate was reduced by MON (*P* < 0.01), but only acetate was further reduced by the combination of

additives ($P < 0.01$), while butyrate was increased by NIT ($P < 0.01$). Propionate molar ratio was increased by MON when compared to control (43.2 vs. 23.8 mol/ 100 mol; $P < 0.01$) and further increased by NIT + MON (46.6 vs. 23.8 mol/ 100 mol; $P < 0.01$). Due to additives effect on acetate and propionate molar proportions, A:P ratio was further reduced by NIT + MON ($P < 0.01$). Isovalerate and isobutyrate molar concentration was not affected by treatments ($P > 0.10$), but valerate was increased by NIT ($P < 0.01$) and reduced by MON ($P < 0.01$) and NIT + MON ($P < 0.01$). Caproate was significantly reduced by MON (0.4 vs. 2.3 mol/ 100 mol; $P < 0.01$) and NIT + MON (0.9 vs. 2.3 mol/ 100 mol; $P < 0.01$).

Additives affected H_2 balance; NIT and NIT + MON significantly reduced H_2 recovery when compared to control (58.5 and 68.5 vs. 74.1%, respectively; $P < 0.01$) and MON increased H_2 recovery (77.5 vs. 74.1%; $P < 0.01$). Metabolic H_2 production was reduced by MON ($P < 0.01$) and H_2 utilized was reduced by NIT and MON when compared to control ($P < 0.01$). Total hexose fermented was increased by NIT at lower dose (45.2 vs. 41.1 μ moles/ mL; $P < 0.01$) and reduced by the combination of additives (37.8 vs. 41.1 μ moles/ mL; $P = 0.02$). Fermentation efficiency was significantly increased by MON when compared to control (84.6 vs. 76.6%; $P < 0.01$) and a tendency for further increase was detected when additives were combined (86.3 vs. 76.6%; $P = 0.07$).

2.5. Discussion

2.5.1. CH_4, N_2O and VFA production

The theoretical potential benefit of NIT and MON to interfere with the ruminal H_2 pool and decrease CH_4 production was confirmed *in vitro* by our studies. Lower and higher doses of NIT reduced CH_4 production compared to control by 57 and 67% in EXP1 and by 54 and 71% in EXP2, respectively. The decrease in CH_4 found in both experiment can be explained by the effect

of the additives on rumen metabolism. Nitrate reduction to NH_3 competes with methanogens in ruminal fluid for H_2 generated during fermentation of carbohydrates. When NIT is reduced to nitrite (**NO_2**) and then to NH_3, the process captures 4 moles of H_2 per mole of NIT reduced (Hino and Asanuma, 2003). Thermodynamically, both reactions involved in NIT reduction to NH_3 are energetically more favorable to occur than reduction of CO_2 by methanogens (Ungerfeld and Kohn, 2003). Decreased CH_4 formation as a result of NIT supplementation has been studied both *in vitro* (Guo et al., 2009; Patra and Yu, 2014) and *in vivo* (van Zijderveld et al., 2011; Newbold et al., 2014) suggesting a consistent effect of NIT as a CH_4 mitigation strategy. Moreover, additive CH_4 mitigation effect of NIT with other nutritional strategies that affect the ruminal H_2 pool, such as supplementation with lipids (Guyander et al., 2015), sulfate (Van Zijderveld et al., 2010) and saponin (Patra and Yu, 2014) have been reported in the literature. In our trials, assuming that all NIT added was reduced to NH_3, CH_4 mitigation observed was explained by NIT reduction pathway by 67% and 68% in EXP1 and EXP2, respectively (actual CH_4 mitigation in moles / theoretical mitigation potential considering that 1 mole of NIT should decrease 1 mole of CH_4 and assuming all NIT was reduced to NH_3). This suggests that NIT or its reduction intermediates may affect the rumen metabolism and methanogenesis by an alternative mechanism (e.g. toxicity to methanogens) other than just competing for reducing equivalents.

A possible alternative explanation for the effect of NIT on ruminal CH_4 decrease is that intermediates of NIT metabolism (NO_2, nitric oxide (**NO**), N_2O) can be toxic to rumen microorganisms. When NIT in the form of a potassium salt (KNO_3) was fed to goats at 6 and 9 g/d, the total number of methanogens and protozoa copy numbers estimated by real-time PCR was significantly reduced (Asanuma et al., 2015). Protozoa are known to be important H_2 producers and their decrease is often associated with a decrease in methanogenesis (Williams

and Coleman, 1997; Morgavi et al., 2012). Feeding lactating cows 3% calcium nitrate ($CaNO_3$) in diet DM with or without linseed oil caused an increase in rumen dissolved H_2 up to 2 h after feeding (Guyander et al., 2015), which according to the authors could be caused by a toxic effect of NIT or its intermediates on H_2 utilizers such as methanogens.

Jones (1971) and Kaspar and Tiedje (1981) reported that in the rumen ecosystem NIT is mainly metabolized by dissimilatory NIT reduction to ammonium, but depending on the balance of enzyme activities, N_2O can be formed via denitrification pathway, which would involve the reduction of NIT to NO_2 followed by the conversion of NO_2 to N_2O or N_2, or a mixture of the two gases. Because the rumen inoculum used in our trials were derived from animals not adapted to NIT, it is possible that NO_2 may have accumulated in the system, and instead of being reduced to NH_3, it diverged to denitrification pathway which is the primary source of N_2O in anaerobic conditions (Sorensen, 1978; Oremland et al., 1984; King and Nedwell, 1985). Wang (2012) also observed significant increases in N_2O levels when NIT was added at 2 and 3% of diet DM during 24-h *in vitro* incubations of ruminal mixed microorganisms. Increased N_2O production was also reported *in vivo* when NIT was supplemented to sheep at 2% of diet DM (de Raphélis-Soissan et al., 2014).

An interaction between NIT and MON resulted in higher levels of N_2O when the additives were combined in EXP2 (up to 75 times greater than control). However, studies have shown that MON does not affect major species of NIT reducers in the rumen. *Selenomonas ruminantium* is a representative NIT and nitrite-reducing ruminal bacterium present at much higher numbers than other nitrate and nitrite-reducers, such as *Veillonella parvula* and *Wolinella succinogenes* (Asanuma et al., 2002; Iwamoto et al., 2002). However, concentrations of MON as high as 40 mg/L did not affect growth of pure cultures of *Selenomonas ruminantium,* and

increases in propionate production commonly found in MON-fed animals may be related to selection of *Bacterioides* and *Selenomonas ruminantium* strains, given the ability of these species to resist higher levels of MON (Chen and Wolin, 1979). *Veillonella parvula* required 14.3 mg/L of culture media of MON to have growth inhibited in pure cultures not adapted to MON (Newbold et al., 1993). This level was more than 2 times our highest dose of MON tested. However, it is possible that variations in animal microbial populations from inoculum donors (Kocherginskaya et al., 2001) could resulted in strains more susceptible to MON and, if MON at the levels tested in our experiments inhibited the growth of a NIT or NO_2 reducer, then NO_2 could accumulate and favor the formation of N_2O. Studies to measure NIT reduction intermediates and microbial population dynamics when MON is fed in combination with NIT are encouraged to clarify the role of different species in NIT metabolism in the rumen and potential effect of MON on growth of such species.

Monensin reduced CH_4 production by approximately 55% in EXP1 and 50% in EXP2. The most consistent and well documented alteration caused by MON on ruminal fermentation is the increased molar proportion of propionate with a concurrent decrease in the molar proportion of acetate and butyrate. The increase in rumen propionate is accompanied by a decrease in the amount of CH_4 production because MON reduce the number of H_2 generating bacteria (Van Nevel and Demeyer, 1977; Wallace et al.,1981), which is supported by the inability of MON to reduce CH_4 production when H_2 and CO_2 are provided (Russell and Martin, 1984). Moreover, during propionate formation fumarate is reduced to succinate and 1 mole of H_2 is consumed in this reaction, resulting in net uptake of H_2 (Ungerfeld and Kohn, 2006). In EXP1 and EXP2 the addition of MON resulted in 48% and 46% increase in propionate molar proportion when

compared to control, partially explaining the decrease in CH_4 production observed when MON was added.

Hydrogen gradients exist in methanogenic ecosystems (Boone et al., 1989) and, in the rumen, a major pathway for propionate synthesis involves the use of H_2 to reduce fumarate to propionate (Ungerfeld and Kohn, 2006). However, kinetic limitations such as availability of H_2 and/or propionate precursors (i.e, fumarate and succinate) may limit propionogenesis because the K_m for H_2 from methanogens is lower than that of five major ruminal species of fumarate reducers (Asanuma et al., 1999). It is possible that NIT + MON coupled with a high concentrate diet in EXP2 further stimulate propionogenesis by reducing kinetically limitations of propionate formation, such as 1) greater H_2 concentration in liquid and gas phase due to NIT effect (van Zijderveld et al., 2011; Guyander et al., 2015); 2) increased proportion of propionate producing species due to MON effect (Van Nevel and Demeyer, 1977; Wallace et al.,1981); and 3) a diet consisting of 90% concentrate that could have supplied enough propionate precursos.

2.5.2. Fermentation balance

Stoichiometric calculations are helpful in describing alterations in fermentation, but they must be interpreted with caution because manipulating fermentation may invoke reactions not considered in the calculations and fermentation uncoupled from cell growth may occur (Chalupa, 1977). It should also be stressed that the approach used in our experiments involves a simplification of very complex systems of feed, animal, and microbial components in a closed, *in vitro* system. However, extensive empirical studies have been conducted in co-cultures of several rumen anaerobic microorganisms and methanogens that validate the use of fermentation balance data to analyze treatment effects (Ørskov et al., 1968; Russell and Hespell, 1981; Demeyer, 1991).

A significant decrease in metabolic H_2 production when compared to control was observed in EXP1 and EXP2 when MON was present (166.4 vs. 191.1 and 166.3 vs 193.8 μ moles/ mL, respectively), a consequence of decreased A:P ratio. Because H_2 utilized was not significantly affected, recovered H_2 was increased by MON. Similar results were found when MON was tested at 0.1, 1 and 5 ppm in 17-h *in vitro* incubations (Richardson et al., 1976). On the other hand, NIT reduced H_2 recovery when compared to control because NIT did not affect production of major VFA while significantly reduced utilization of metabolic H_2 through CH_4 formation.

In rumen studies, fermentation efficiency can be defined as a measure of the energy present in a fermentable substrate (i.e. hexoses and amino acids) that is recovered in usable forms to the host animal, such as VFA and microbial protein. In this study, fermentation efficiency was increased by MON addition and tended to further increase in EXP2 due to the combination of additives at lower doses. Production of propionate is energetically more efficient than other major VFA. Because of differences in the production and utilization of metabolic H_2, the efficiencies of fermenting hexose to acetate, propionate and butyrate are 62, 109 and 78%, respectively (Orskov and Ryle, 1990). Theoretically, if 1 mole of hexose is fermented to 2 moles of propionate then no H_2 and CO_2 are lost (Wolin et al., 1997), while in the same process one mole of H_2 is incorporated during propionate formation, as discussed before. Therefore, treatments that enhance propionate, and to a lesser extent butyrate, at the expense of acetate are likely to increase fermentation efficiency.

2.5.3. IVDMD

A significant effect of treatments when compared to control on IVDMD were only detected when NIT + MON were combined at higher doses in EXP1 (31.7 vs. 37.5 %) and EXP2 (76.6 vs. 79.9 %). The lack of effect of NIT on IVDMD is consistent with results found by Patra and Yu

(2014), when NIT was added at 5 mM to rumen batch culture containing alfalfa hay and dairy concentrate feed (50:50) as substrate (higher and lower doses in our trials resulted in 4 and 2 mM of NIT, respectively). Similarly, Maraes et al. (1988) observed that NIT (as KNO_3) affected the rate but not the extent of digestion in 72-h fermentation, while NO_2 significantly reduced IVDMD and cellulolytic and xylanolytic microbial growth rates. In the latter study, the effect of NIT on digestion rate was suggested to be linked with accumulation of NO_2 in the media, which peaked at about 18 h. Therefore, NO_2 is likely the primary factor reducing digestibility of diets high in NIT. Adaptation of rumen microbes to NIT presence increase the activity of species able to reduce NIT to NH_3, reducing negative effects of ruminal NO_2 accumulation on animal health and diet digestibility (Lee and Beauchemin, 2014), and by NO_2 effect on growth of major cellulolytic bacteria (Maraes et al., 1988; Zhou et al., 2012), respectively.

The absence of effect of MON on IVDMD in EXP1 (39.5 vs. 37.5 %) and EXP2 (81.1 vs. 79.9) is consistent with results of Duff et al. (1994) who found no effect of MON at 4 mg/L in 24-h IVDMD of a 90% concentrate substrate. Similarly, when studying the effect of MON (5 mg/L) on substrates containing different levels of S (0.2 to 0.8 % of DM), no effect on IVDMD was detected by Quinn et al. (2009) and Smith et al. (2014). Van Nevel and Demeyer (1977) reported no effect of MON (5 mg/L) on the quantity of substrate fermented despite a decrease in microbial growth, suggesting that MON uncouples microbial growth from fermentation. Although we did not measure microbial growth, total VFA production (mM) was not affected by MON on EXP1 (46.8 vs. 48.5 mM) and EXP2 (72.3 vs. 66.7), suggesting that total microbial growth was not affected, even though specific specie populations were likely changed (e.g. inhibition of acetate-producing bacteria and protozoa).

2.5.4. The potential to use nitrate as a feed additive for ruminants

Nitrates may be present in the diet of ruminants in a continuous basis, usually in the form of nitrate salts and ammonium nitrate. The most common source are forages from the perennial rye grass and sorghum family (sorghum, sudan, pearl millet and their crosses; Leng and Preston, 2010) and factors affecting NIT concentration in forage have been well demonstrated by Leng (2008), and include forage maturity, soil conditions (e.g., moisture content), and application of fertilizers to soil. The majority of research conducted in the past focused on understanding the metabolism of NIT in the rumen in order to avoid poisoning, which is caused when the rumen microbes are not adapted to NIT and NO_2 accumulates in the rumen, ultimately being absorbed into the blood (Lee and Beauchemin, 2014). However, recent studies have shown the potential of NIT to consistently reduce CH_4 levels (see previous discussion on CH_4) without resulting in toxicity. In addition, NIT serves as a valuable source of NPN to microorganisms, with recent studies reporting that NIT may increase microbial N output in isonitrogenous diets when compared to urea (Guo et al., 2009; Guyader et al., 2017). An encapsulated form of NIT was also developed (El-Zaiat et al., 2014) to control the release rate of NIT in the rumen, allowing the microbes to adapt to higher NIT levels and therefore reducing potential toxicity in commercial settings. Despite the consistent results showing the benefits of supplementing ruminants with NIT as a CH_4 mitigation strategy and as a source of NPN, the authors are not aware of any regulations or recommendations for producers in the USA to use NIT as a feed additive.

A potential model to be used in the USA is the case of Australia where carbon credits are now available for managed feeding of NIT for grazing ruminants (Department of Environment, 2015). Acceptance of NIT feeding by producers as a means of gaining carbon credits from

reduced CH_4 output by their livestock will depend on whether they can achieve the nutritional benefits of NPN supplementation using NIT at a cost similar to that of urea supplementation, and with a minimal risk of NO_2 poisoning or other adverse effects on animal production. (Nolan et al., 2016). Because MON is a widely used additive in the USA, especially in finishing beef cattle, more *in vivo* studies looking at the interaction of both additives are needed before further recommending their combination.

2.6. Conclusion

Overall, the combination of NIT + MON in two contrasting diets showed beneficial effects on ruminal fermentation *in vitro* by reducing CH_4 formation while not affecting IVDMD and VFA production. However, NIT increased N_2O production which partially offset the benefit of CH_4 decrease on total greenhouse gas balance. The VFA profile was mainly affected by MON presence as a consequence of its effect on ruminal microbiota which favors more efficient fermentation pathways. The combination of additives at lower doses further increased propionate levels in a high concentrate diet, demonstrating a potential capacity of propionogenesis to uptake additional H_2 present in ruminal fluid and convert it into a highly reduced VFA. The combination of NIT and MON may serve as a dietary means of reducing CH_4 generated during ruminants foregut fermentation. More studies are encouraged to better understand the effect of the additives combination in ruminal fermentation including effect on NIT reduction intermediates, animal performance and metabolism.

Table 2.1. Ingredients and chemical composition of experimental diets for *in vitro* EXP1 (100:0 roughage: concentrate) and EXP2 (10:90 roughage: concentrate)[1].

	0 NIT			1.25 NIT			2.5 NIT		
Item (DM, % unless otherwise stated)	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON
EXP1									
Grass hay	97.5	97.5	97.5	97.0	97.0	97.0	97.0	97.0	97.0
EXP2									
Grass hay	9.8	9.8	9.8	9.7	9.7	9.7	9.7	9.7	9.7
Ground corn	87.7	87.7	87.7	87.5	87.5	87.5	87.5	87.5	87.5
EXP1 and EXP2									
Nitrate source[2]				1.5	1.5	1.5	2.9	2.9	2.9
Monensin, mg/L[3]		3	6		3	6		3	6
Urea	1.3	1.3	1.3	0.7	0.7	0.7			
Limestone	1.2	1.2	1.2	0.6	0.6	0.6			
Calculated chemical composition (% of DM)									
EXP1									
DM	90.7	90.7	90.7	90.7	90.7	90.7	90.7	90.7	90.7
CP	12.4	12.4	12.4	12.5	12.5	12.5	12.3	12.3	12.3
NDF	65.1	65.1	65.1	65.1	65.1	65.1	65.1	65.1	65.1
NFC	15.3	15.3	15.3	15.3	15.3	15.3	15.3	15.3	15.3
Ash	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1
Ca	1.1	1.1	1.1	1.2	1.2	1.2	1.2	1.2	1.2
EXP2									
DM	91.1	91.1	91.1	91.1	91.1	91.1	91.1	91.1	91.1

Table 2.1 (cont'd)

CP	11.8	11.8	11.8	11.9	11.9	11.9	11.7	11.7	11.7
NDF	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3
NFC	68.9	68.9	68.9	68.9	68.9	68.9	68.9	68.9	68.9
Ash	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Ca	0.5	0.5	0.5	0.6	0.6	0.6	0.6	0.6	0.6

[1]NIT = Calcium nitrate (0, 1.25 and 2.5% of diet DM); MON = Sodium monensin (0, 3 and 6 mg/L).

[2]Nitrate source was manufactured by Yara International, Oslo, Norway. The source of nitrate was the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$]; 85.6% DM; 17.6% N, 19.6% Ca, and 71.4% nitrate on a DM basis.

[3]Monensin sodium ($C_{36}H_{61}NaO_1$; Sigma Aldrich Corporation, St. Louis).

Table 2.2. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* gas and CH_4 production in EXP1 (100:0 roughage:concentrate).

	0 NIT			1.25 NIT			2.5 NIT			
Item	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	Pooled SEM
pH	6.2	6.2	6.2	6.2	6.2	6.2	6.3	6.3	6.2	0.2
IVDMD, %	37.5^{abc}	40.0^{a}	39.3^{ab}	37.3^{abc}	35.6^{bcd}	34.7^{cd}	38.4^{abc}	35.6^{cd}	31.7^{d}	0.7
Gas production, mL										
Gas (0-4h)	21.69^{a}	18.60^{b}	19.23^{b}	21.28^{a}	17.79^{b}	18.2^{b}	21.8^{a}	18.53^{b}	19.54^{b}	0.9
Gas (4-8h)	16.35^{a}	14.03^{bc}	13.04^{c}	15.06^{a}	13.2^{c}	13.48^{c}	15.24^{a}	13.89^{c}	13.73^{c}	0.3
Gas (8-12h)	12.18^{a}	10.46^{b}	9.72^{c}	10.62^{b}	10.02^{bc}	9.16^{c}	10.51^{b}	9.6^{c}	9.35^{c}	0.5
Gas (12-24h)	22.5^{a}	17.71^{b}	15.83^{bc}	21.11^{a}	13.56^{c}	12.57^{c}	20.42^{a}	12.48^{c}	11.45^{c}	0.4
Gas (12-48h)	21.94^{a}	20.48^{a}	18.32^{b}	20.73^{a}	18.46^{b}	17.3^{b}	19.07^{ab}	18.13^{b}	12.7^{d}	0.6
Total gas	94.66^{a}	81.26^{c}	76.15^{d}	88.81^{b}	73.04^{d}	70.72^{de}	87.04^{b}	72.43^{de}	66.77^{e}	0.8
CH_4 production, mL										
CH_4 (0-4h)	0.1^{a}	0.08^{b}	0.08^{b}	0.08^{b}	0.06^{c}	0.07^{b}	0.1^{a}	0.09^{a}	0.07^{b}	0.02
CH_4 (4-8h)	0.85^{a}	0.45^{b}	0.23^{c}	0.45^{b}	0.16^{d}	0.16^{d}	0.4^{b}	0.13^{d}	0.19^{c}	0.06

Table 2.2 (cont'd)

CH_4 (8-12h)	1.41[a]	0.48[b]	0.57[b]	0.5[b]	0.21[c]	0.21[c]	0.57[b]	0.18[c]	0.22[c]	0.06
CH_4 (12-24h)	2.99[a]	1.61[b]	1.24[b]	0.66[c]	0.33[d]	0.21[d]	0.73[c]	0.22[d]	0.15[d]	0.2
CH_4 (24-48h)	3.8[a]	1.34[c]	2.24[b]	2.24[b]	0.82[d]	0.67[d]	1.22[c]	0.31[d]	0.17[e]	0.15
Total CH_4	9.17[a]	3.98[b]	4.38[b]	3.95[b]	1.58[cd]	1.33[cd]	3.03[bc]	0.95[d]	0.81[d]	0.2
CH_4, µM	346.7[a]	150.6[b]	165.4[b]	149.4[b]	59.8[d]	50.1[d]	114.7[c]	35.9[e]	30.5[e]	13.8
CH_4, mL/g DM	18.3[a]	7.9[b]	8.7[b]	7.9[b]	3.1[cd]	2.6[cd]	6.0[bc]	1.9[d]	1.6[d]	0.5
CH_4, mL/g DMD[2]	38.4[a]	20.6[c]	17.3[c]	31.6[b]	4.5[e]	3.3[e]	11.4[d]	3.1[e]	4.3[e]	0.7
N_2O, mL×10^{-4}	1.2[c]	1.2[c]	1.2[c]	12.6[b]	17.0[b]	16.7[b]	23.3[a]	26.2[a]	25.1[a]	0.8

[1]NS indicates P > 0.10, * indicates P < 0.1 and ** indicates P < 0.05.

[2]Dry matter digestibility.

[a,b,c,d,e] Within a row, means without a common superscript letter differ, P < 0.05.

Table 2.3. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* VFA profile and fermentation balance in EXP1 (100:0 roughage:concentrate)[1].

	0 NIT			1.25 NIT			2.5 NIT			
Item	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	Pooled SEM
Total VFA, mM[1]	48.5[a]	47.5[ab]	46.1[ab]	49.2[a]	46.4[b]	45.3[b]	47.1[ab]	45.9[b]	44.6[b]	4.6
VFA composition, mol/100 mol										
Acetate (A)	62.6[a]	48.6[b]	48.2[b]	61.4[a]	49.3[b]	48.2[b]	63.3[a]	48.5[b]	49.3[b]	4.7
Propionate (P)	22.5[b]	40.2[a]	40.5[a]	21.1[b]	39.4[a]	40.8[a]	18.6[b]	41.5[a]	39.4[a]	2.1
Isobutyrate	1.4	1.2	1.5	1.6	1.1	1.0	1.7	1.4	1.4	0.8
Butyrate	9.2[b]	5.8[c]	6.1[c]	10.4[a]	6.3[c]	5.8[c]	10.6[a]	5.1[c]	4.7[c]	0.3
Isovalerate	0.9	1.2	1.1	0.8	0.6	0.7	1.2	1.1	0.8	0.06
Valerate	2.5	1.8	1.5	2.8	2.1	2.2	3.1	1.5	2.7	0.5
Caproate	1.2	1.5	1.4	2.1	1.3	1.4	2.2	1.2	1.4	0.1
A:P	2.8[a]	1.2[b]	1.1[b]	2.9[a]	1.2[b]	1.1[b]	3.4[a]	1.1[b]	1.2[b]	0.05
Metabolic hydrogen balance (μ moles/ mL of fermentation media)[3]										
Produced	191.1[a]	166.6[b]	166.2[b]	192.7[a]	168.6[b]	166.2[b]	196.2[a]	164.1[b]	163.8[b]	12.1
Utilized	102.3[ab]	109.4[a]	111.9[a]	81.4[c]	99.5[b]	100.6[b]	73.5[d]	98.3[b]	94.1[b]	7.3
Recovered, %	53.5[bc]	65.6[a]	67.3[a]	42.4[d]	59.0[b]	60.5[ab]	37.4[d]	59.9[b]	57.4[b]	6.4
Hexose fermented	27.1	26.0	25.9	27.2	26.3	26.2	27.3	25.8	25.8	2.5

Table 2.3 (cont'd)

Fermentation efficiency, %	70.9[b]	78.7[a]	79.0[a]	69.7[b]	78.6[a]	79.1[a]	68.4[b]	79.4[a]	77.4[a]	6.5

[1]VFA values reflect net production.

[2]NS indicates P > 0.10, * indicates P < 0.1 and ** indicates P < 0.05.

[3]Stoichiometric calculations were based upon balances applied to rumen fermentation (Wolin, 1960; Demeyer and Tamminga, 1987; Demeyer, 1991).

[a,b,c,d] Within a row, means without a common superscript letter differ, P < 0.05.

Table 2.4. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* gas and CH_4 production in EXP2 (10:90 roughage:concentrate).

	0 NIT			1.25 NIT			2.5 NIT			
Item	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	Pooled SEM
pH	6.1	6.3	6.3	6.2	6.2	6.4	6.2	6.3	6.3	0.2
IVDMD, %	79.9^{ab}	80.4^{ab}	81.9^{ab}	77.4^{bc}	82.1^{a}	79.2^{abc}	78.9^{abc}	77.2^{bc}	76.6^{c}	0.6
Gas production, mL										
Gas (0-4h)	23.54^{b}	18.82^{c}	18.62^{c}	26.09^{a}	22.99^{b}	20.25^{bc}	22.89^{b}	23.04^{b}	23.62^{b}	2.3
Gas (4-8h)	48.67^{a}	38.21^{b}	38.04^{b}	47.4^{a}	37.83^{b}	36.44^{b}	40.92^{bc}	38.57^{b}	41.11^{c}	4.5
Gas (8-12h)	46.79^{a}	36.21^{bc}	34.77^{c}	43.94^{ab}	34.97^{c}	31.84^{c}	42.61^{ab}	28.44^{d}	28.85^{d}	3.7
Gas (12-24h)	45.29^{a}	47.51^{a}	44.65^{a}	42.31^{b}	43.47^{ab}	42.55^{b}	40.51^{b}	44.8^{a}	38.45^{c}	3.8
Gas (12-48h)	32.64^{a}	29.99^{ab}	31.02^{a}	31.05^{a}	24.92^{c}	27.72^{b}	24.82^{c}	27.56^{b}	27.58^{b}	3.0
Total gas	196.92^{a}	170.74^{bc}	167.0^{bcd}	190.8^{a}	164.2^{bcd}	158.79^{d}	172.76^{b}	162.4^{bcd}	159.6^{cd}	6.1
CH_4 production, mL										
CH_4 (0-4h)	0.46^{a}	0.29^{b}	0.18^{c}	0.55^{a}	0.21^{bc}	0.17^{c}	0.29^{b}	0.18^{c}	0.26^{b}	0.05
CH_4 (4-8h)	1.87^{a}	0.77^{c}	0.75^{c}	1.05^{b}	0.17^{e}	0.19^{e}	0.4^{d}	0.22^{e}	0.52^{d}	0.07
CH_4 (8-12h)	2.63^{a}	1.42^{b}	1.32^{b}	1.36^{b}	0.17^{d}	0.11^{d}	0.34^{c}	0.18^{d}	0.23^{d}	0.1
CH_4 (12-24h)	3.95^{a}	2.39^{b}	2.19^{b}	3.8^{a}	0.5^{cd}	0.29^{d}	0.88^{c}	0.21^{d}	0.29^{d}	0.14

Table 2.4 (cont'd)

CH_4 (24-48h)	6.42[a]	3.42[c]	2.48[d]	5.5[b]	0.8[e]	0.54[e]	2.6[d]	0.49[e]	0.33[e]	0.2
Total CH_4	15.3[a]	8.3[c]	6.9[c]	12.2[b]	1.8[e]	1.3[e]	4.5[d]	1.2[e]	1.6[e]	0.2
CH_4, µM	580.1[a]	314.3[c]	262.2[c]	463.8[b]	70.3[e]	49.5[e]	170.9[d]	48.4[e]	62.3[e]	19.2
CH_4, mL/g DM	30.7[a]	16.6[c]	13.8[c]	24.5[b]	3.7[e]	2.6[e]	9.0[d]	2.5[e]	3.3[e]	0.5
CH_4, mL/g DMD[2]	38.4[a]	20.6[c]	17.3[c]	31.6[b]	4.5[e]	3.3[e]	11.4[d]	3.1[e]	4.3[e]	0.7
N_2O, mL×10^{-4}	0.8[d]	0.7[d]	0.7[d]	9.7[d]	28.5[c]	30.7[bc]	44[b]	76.3[a]	71.5[a]	2.1

[1]NS indicates P > 0.10, * indicates P < 0.1 and ** indicates P < 0.05.

[2]Dry matter digestibility.

[a,b,c,d,e] Within a row, means without a common superscript letter differ, P < 0.05.

Table 2.5. Effect of nitrate (NIT; 0, 1.25 and 2.5 g/ 100g of DM), monensin (MON; 0, 3 and 6 mg/L) and their combination on *in vitro* VFA profile and fermentation balance in EXP2 (10:90 roughage:concentrate)[1].

	0 NIT			1.25 NIT			2.5 NIT			
Item	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	0 MON	3 MON	6 MON	Pooled SEM
Total VFA, mM[1]	66.7[bc]	71.5[a]	73.2[a]	74.3[a]	65.7[c]	68.5[bc]	63.2[d]	68.6[bc]	66.6[bc]	2.2
VFA composition, mol/100 mol										
Acetate (A)	54.2[a]	43.6[c]	44.9[c]	54.9[a]	39.2[e]	38.6[e]	48.9[b]	41.4[d]	42.3[d]	0.5
Propionate (P)	23.8[c]	43.4[b]	43.0[b]	23.6[c]	49.4[a]	47.8[a]	26.9[c]	45.8[ab]	43.4[b]	1.1
Isobutyrate	2.8	3.4	1.6	3.0	0.6	2.4	3.3	1.4	3.5	0.8
Butyrate	15.2[b]	7.0[d]	7.5[cd]	14.7[b]	7.9[c]	7.8[c]	17.4[a]	8.3[c]	7.7[c]	0.3
Isovalerate	3.1	2.8	2.6	3.0	2.5	2.7	2.5	2.7	2.7	0.1
Valerate	6.6[b]	4.2[c]	4.0[c]	6.2[b]	4.2[c]	4.5[c]	9.1[a]	4.7[c]	4.3[c]	0.3
Caproate	2.3[a]	0.3[c]	0.4[c]	1.4[b]	0.7[c]	2.0[a]	2.1[a]	0.5[c]	0.4[c]	0.07
A:P	2.3[a]	1.0[c]	1.0[c]	2.3[a]	0.8[d]	0.8[d]	1.8[b]	0.9[cd]	1.0[c]	0.05
Metabolic hydrogen balance (µ moles/ mL of fermentation media)[3]										
Produced	193.8[a]	164.9[b]	167.8[b]	194.5[a]	164.2[b]	158.1[c]	193.8[a]	167.0[b]	164.7[b]	6.8
Utilized	143.7[a]	130.6[b]	127.3[b]	125.8[b]	119.0[c]	111.0[c]	101.9[d]	110.9[c]	107.2[c]	6.1
Recovered, %	74.1[a]	79.1[a]	75.9[ab]	64.6[c]	72.4[b]	70.2[bc]	52.5[d]	66.4[bc]	65.0[c]	3.4

Table 2.5 (cont'd)

Hexose fermented	41.1^{b}	39.4^{b}	40.9^{b}	45.2^{a}	37.3^{c}	38.3^{bc}	40.9^{b}	39.0^{b}	36.7^{c}	4.1
Fermentation efficiency, %	76.6^{c}	84.8^{b}	84.4^{b}	76.4^{c}	87.3^{a}	87.1^{a}	78.5^{c}	85.9^{ab}	85.1^{ab}	6.7

[1]VFA values reflect net production.

[2]NS indicates P > 0.10, * indicates P < 0.1 and ** indicates P < 0.05.

[3]Stoichiometric calculations were based upon balances applied to rumen fermentation (Wolin, 1960; Demeyer and Tamminga, 1987; Demeyer, 1991).

[a,b,c,d] Within a row, means without a common superscript letter differ, P < 0.05.

LITERATURE CITED

LITERATURE CITED

Asanuma, N., S. Yokoyama, and T. Hino. 2015. Effects of nitrate addition to a diet of fermentation and microbial populations in the rumen of goats, with special reference to Selenomonas ruminantium having the ability to reduce nitrate and nitrite. Anim. Sci. J. 86: 378–384.

Asanuma, N., M. Iwamoto, M. Kawato and T. Hino. 2002. Numbers of nitrate-reducing bacteria in the rumen as estimated by competitive polymerase chain reaction. Anim. Sci. J. 73: 199–205.

Asanuma, N., M. Iwamoto, and T. Hino. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms in vitro. J. Dairy Sci. 82: 780–787.

Blaxter, K.L. 1966. The energy metabolism of ruminants. 2nd. ed. Thomas Publisher. Springfield, Ill.

Boone, D. R., R. L. Johnson, and Y. Liu. 1989. Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of Km for H_2 or formate uptake. Appl. Environ. Microbiol. 55: 1735–1741.

Chalupa, W. 1977. Manipulating rumen fermentation. J. Anim. Sci. 45:585–599.

Chen, M., and M. J. Wolin. 1979. Effect of monensin and lasalocid sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. 38:72–77.

Demeyer, D.I. 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut. In: J. P. Jouany, editor, Rumen Microbial Metabolism and Ruminant Digestion. INRA editions, Paris, France. p. 217–237.

Demeyer, D.I. and S. Tamminga. 1987. Microbial protein yield and its prediction. In: R. Jarrige and G. Alderman, editors, Feed Evaluation and Protein Requirement Systems for Ruminants. Office for Official Publications of the European Communities, Luxembourg City, Luxembourg. p. 129–141.

Department of Environment, Australian Government. 2015. Carbon farming initiative. Reducing Greenhouse Gas Emissions in Beef Cattle by Feeding Nitrate Supplements. http://www.environment.gov.au/system/files/pages/43b17857-4fc0-4ad8-884c-8a1d6d9a7732/files/doic-decision-2013ld054.pdf. (Accessed 1 July 2017).

Duff, G. C., M. L. Galyean, and M. E. Branine. 1994. Effects of adaptation to lasalocid, monensin or a daily rotation of lasalocid and monensin on in vitro fermentation of a 90% concentrate diet. Can. J. Anim. Sci. 75:129–134.

El-Zaiat, H. M., R. C. Araujo, Y. A. Soltan, A. S. Morsy, H. Louvandini, A. V. Pires, H. O. Patino, P. S. Correa, and A. L. Abdalla. 2014. Encapsulated nitrate and cashew nut shell

liquid on blood and rumen constituents, methane emission, and growth performance of lambs. J. Anim. Sci. 92: 2214-24.

Gerber, P. J., H. Steinfeld, B. Henderson, A. Mottet, C. Opio, J. Dijkman, A. Falcucci, and G. Tempio. 2013. Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations, Rome, Italy.

Goering, H. K. and P. J. Van Soest. 1970. Forage and fiber analysis (apparatus, reagents, procedures, and some applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC .

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

Guo, W. S., D. M. Schaefer, X. X. Guo, L. P. Ren, and Q. X. Meng. 2009. Use of nitrate-nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis in vitro. Asian-Australas. J. Anim. Sci. 22:542–549.

Guyader, J., M. Eugène, B. Meunier, M. Doreau, D. P. Morgavi, M. Silberberg, Y. Rochette, C. Gerard, C. Loncke, and C. Martin. 2015. Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. J. Anim. Sci. 93:3564–3576.

Guyader J., E.M. Ungerfeld, and K.A. Beauchemin. 2017. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). Front. Microbiol. 8:393.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press Publisher. New York, NY.

Iwamoto, M., N. Asanuma and T. Hino. 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. 8:209–215.

Johnson, K.A and D.E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492.

Kaspar, H. F. and J. M. Tiedje. 1981. Dissimilatory reduction of nitrate and nitrite in the bovine rumen: nitrous oxide production and effect of acetylene. Appl. Environ. Microbiol. 41:705–709

King, D. and D.B. Nedwell. 1985. The influence of nitrate concentration upon the end product of nitrate dissimilation by bacteria in anaerobic salt-marsh sediment. FEMS Microbiol. Ecol. 31:23–28.

Kocherginskaya, S. A., I.A. Rustam, and B. A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. Anaerobe. 7: 119–134.

Lee, C. and K. A. Beauchemin. 2014. A review of feeding supplementary nitrate to ruminant animals: Nitrate toxicity, methane emissions, and production performance. Can. J. Anim. Sci. 94: 557–570.

Leng, R. A. 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A report to the department of climate change. Commonwealth Government of Australia, Canberra. www.penambulbooks.com/Downloads/Leng-Final%20 Modified%20%2017-9-2008.pdf (Accessed 14 May 2017.)

Leng, R. A. and T. R. Preston. 2010. Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. Livestock Research for Rural Development; Volume 22, article no. 221. http://www. lrrd.org/lrrd22/12/leng22221.htm. (Accessed January 26, 2017.)

Marais, J. P., J. J. Therion, R. I. Mackie, A. Kistner, and C. Dennison. 1988. Effect of nitrate and its reduction products on the growth and activity of the rumen microbial population. Br. J. Nutr. 59: 301-13.

Martin, C., D.P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: From microbe to the farm scale. Animal. 4:351–365.

Miller, T.L. 1995. Ecology of methane production and hydrogen sinks in the rumen. In: W.V. Engelhardt, S. Leonhard-Marek, G. Breves, and D. Giesecke, editors, Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. Ferdinand Enke Verlag Publisher, Stuttgart, Germany. p. 317–331.

Morgavi, D. P., C. Martin, J. P. Jouany, and M. J. Ranilla. 2012. Rumen protozoa and methanogenesis: Not a simple cause effect relationship. Br. J. Nutr. 107:388–397.

Newbold, C. J., R. J. Wallace, and N. D. Walker. 1993. The effect of tetronasin and monensin on fermentation, microbial numbers and the development of ionophore-resistant bacteria in the rumen. J. Appl. Microbiol. 75:129–34

Newbold, J. R., S. M. van Zijderveld, R. B. A. Hulshof, W. B. Fokkink, R. A. Leng, P. Terencio, W. J. Powers, P. S. J. van Adrichem, N. D. Paton, and H. B. Perdok. 2014. The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. J. Anim. Sci. 92:5032–5040.

Nolan, J. V., I. R. Godwin, V. de Raph lis-Soissan, and R. S. Hegarty. 2016. Managing the rumen to limit the incidence and severity of nitrite poisoning in nitrate-supplemented ruminants. Anim. Prod. Sci. 56: 1317–1329.

Quinn, M. J., M. L. May, K. E. Hales, N. DiLorenzo, J. Leibovich, D. R. Smith, and M. L. Galyean. 2009. Effects of ionophores and antibiotics on in vitro hydrogen sulfide production, dry matter disappearance, and total gas production in cultures with a steam-flaked corn-based substrate with or without added sulfur. J. Anim. Sci. 87:1705–1713.

Oremland, R.S., C. Umberger, C.W. Culbertson, and R.L. Smith. 1984. Denitrification in San-Francisco Bay intertidal sediments. Appl. Environ. Microbiol. 47:1106–1112.

Ørskov, E. R., W. P. Flatt, and P. W. Moe 1968. Fermentation balance approach to estimate extent of fermentation and efficiency of volatile fatty acid formation in ruminants. J. Dairy Sci. 51: 1429-1435.

Ørskov, E. R. and M. Ryle. 1990. Energy nutrition in ruminants. 1st. Ed. Elsevier Science Publishers, London, UK.

Patra, A. K. and Z. Yu. 2014. Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures in vitro while not adversely affecting feed digestion, fermentation or microbial communities. Bioresour. Technol. 155:129–135.

Russell, J. B. and R. B. Hespell. 1981. Microbial rumen fermentation. J. Dairy Sci. 64: 1153-1169.

Russell, J. B. and S. A. Martin. 1984. Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms in vitro. J. Anim. Sci. 59:1329–1338.

Smith, D. R., N. DiLorenzo, J. Leibovich, M. L. May, M. J. Quinn, J. W. Homm, and M. L. Galyean. 2010. Effects of sulfur and monensin concentrations on in vitro dry matter disappearance, hydrogen sulfide production, and volatile fatty acid concentrations in batch culture ruminal fermentations. J. Anim. Sci. 88: 1503–1512.

Sorensen, J. 1978. Capacity for denitrification and reduction of nitrate to ammonia in a coastal marine sediment. Appl. Environ. Microbiol. 35:301–305.

Ungerfeld, E. M. and R. A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: K. Sejrsen, T. Hvelplund, and M. O. Nielsen, ed. Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 55–85.

US EPA. 2006. Global Anthropogenic Emissions of Non-CO2 Greenhouse Gases: 1990–2020. EPA Report 430-R-06–003. http://www.epa.gov/methane/pdfs/GreenhouseGasReport.pdf Accessed June 1, 2017.

Van Nevel, C. J. and D. I. Demeyer. 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. 34:251–257.

Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93:5856–5866.

Van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94: 4028–4038.

Zhou, Z., Z. Yu. and Q. Meng. 2012. Effects of nitrate on methane production, fermentation, and microbial populations in in vitro ruminal cultures. Bioresource Technol. 103: 173–179.

Wallace, R. J., J. W. Czerkawski, and G. Breckenridge. 1981. Effect of monensin on the fermentation of basal rations in the rumen simulation technique (Rusitec). Br. J. Nutr. 46:131–148.

Wang, Q. 2012. Nutritional Strategies to Mitigate Greenhouse Gas Emissions From Cattle Production. PhD Diss. Univ. of California, Davis.

Williams, A. G. and G.S. Coleman. 1992. The Rumen Protozoa. Springer Verlag Publisher. New York, NY.

Wolin, M. J. 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43:1452–1459.

Wolin, M.J., T.L. Miller, and C. S. Stewart. 1997. Microbe–microbe interaction. In: P.N. Hobson and C.S. Stewart, editors, The Rumen Microbial Ecosystem. 2nd. Ed. Blackie Academic & Professional, London, UK. p. 467–491.

CHAPTER 3

THE EFFECT OF ENCAPSULATED NITRATE AND MONENSIN ON RUMINAL FERMENTATION USING A SEMI-CONTINUOUS CULTUR

The effect of encapsulated nitrate and monensin on ruminal fermentation using a semi-continuous culture system[1]

M. Capelari,§ K. A. Johnson,* B. Latack,§ J. Roth,§ and W. Powers,†[2]

§ Michigan State University, East Lansing, MI, 48824
* Washington State University, Pullman, WA, 99164
† University of California, Oakland, CA, 94607.

[1]The authors thank CAPES foundation, Ministry of Education of Brazil, for providing funds through scholarship number 13634/2013-5, and GRASP Ind. & Com. LTDA/EW|Nutrition GmbH and Elanco Animal Health for additives donation.

[2] Corresponding author: wendy.powers@ucop.edu

3.1. Abstract

Seven 37-d trials tested the effect of encapsulated nitrate (EN) and sodium monensin (MON) in diets commonly fed to dairy (DAIRY; 50:50 forage to concentrate; 4 trials) and beef cattle (BEEF; 15:85 forage to concentrate; 3 trials) on rumen fermentation and methane (CH_4) production using a semi-continuous fermentation system. A 3 × 2 factorial arrangement was used and additives (0, 1.25 and 2.5% of EN; 0 and 4 mg/L of MON) were tested alone and combined (EN+MON) totaling 6 treatments. Rumen fluid was pooled from 5 non-adapted lactating cows fed 50:50 forage to concentrate diet 3 h after morning feeding, and 1 L of processed inoculum was transferred to each 2.2-L vessels. Treatment diets were added to nylon bags which remained in the anaerobic fermentation system for 48 h. Nitrate reduced CH_4 production in DAIRY (24.7 vs. 32.1 mM/d; $P < 0.01$) and BEEF trials (33.5 vs. 43.5 mM/d; $P < 0.01$). Methane production was reduced by MON in DAIRY (26.3 vs. 32.1; $P < 0.01$) and BEEF (26.6 vs. 43.5 mM/d; $P < 0.01$). The combination of EN+MON further reduced CH_4 in DAIRY (21.3 vs. 32.1 mM/ d; $P = 0.03$) and BEEF (19.3 vs 43.5 mM/d; $P = 0.01$). Nitrate did not affect major VFA in DAIRY and BEEF trials, but reduced digestion of protein (96.8 vs. 97.6 %; $P < 0.01$) and starch (79.0 vs. 80.4 %; $P < 0.01$) in DAIRY and NDF (29.3 vs. 32.5 %; $P < 0.01$) and starch (88.5 vs. %; $P < 0.01$) in BEEF. Monensin affected VFA pattern with an increase in propionate ($P < 0.01$) and reduction on acetate ($P < 0.01$) production with consequent reduction on acetate to propionate ratio in DAIRY (1.6 vs. 2.0; $P < 0.01$) and BEEF (1.6 vs. 1.9; $P < 0.01$). Monensin reduced NDF digestion in BEEF only (29.3 vs 32.5 %; $P < 0.01$). Significant concentrations of nitrate and nitrite were detected only for EN and EN+MON ($P < 0.01$). Nitrate and monensin effectively reduced CH_4 production when fed separately and the combination of additives additively reduced CH_4 production. However, additives may impair ruminal digestion of some chemical fractions. *In vivo* studies could better explain animal response and confirm mitigation benefits.

Key words: *in vitro*, methane, monensin, nitrate**,** rumen

3.2. Introduction

Enteric methane (**CH_4**) production represents energy loss to the animal and a source of pollution. Microbial fermentation in the rumen is an oxidative process during which reduced cofactors are re-oxidized through dehydrogenation reactions that release hydrogen (**H_2**) in the rumen. The H_2 is used by methanogenic archaea to reduce carbon dioxide (**CO_2**) into CH_4 (Morgavi et at., 2010), an evolutionary essential reaction in the rumen ecosystem that prevents H_2 accumulation. However, livestock contribute with 6.3% of total anthropogenic GHG emissions, with CH_4 emissions from enteric fermentation accounting for 2.1 Gt CO_2 Eq/yr and manure management accounting for 0.99 Gt CO_2 Eq/yr (IPCC, 2014). In addition, CH_4 represents a loss of 2 – 12% of diet GE (Johnson and Johnson, 1995) that could be rechanneled towards usable products for the host (Grainger and Beauchemin, 2011). Therefore, assuming the efficiency of ruminal metabolism is not compromised, reducing CH_4 production by ruminants contributes to global efforts to reduce GHG emissions and can improve the efficiency of feed use.

Previous work by Capelari and Powers (2017) demonstrated an additive effect of nitrate and monensin (**MON**) on reduction of CH_4 production, *in vitro*. The observed reduction on CH_4 output occurred independently of diet (grass hay only and 90:10 ground corn: grass hay) with absolute CH_4 mitigation averaging 90% when additives were combined. The effect of additives on CH_4 can be explained by the modifications on rumen metabolism, specially H_2 dynamics. Microbes that reduce nitrate to ammonia (**NH_3**) in the rumen compete with methanogens to scavenge H_2 (Leng, 2008) and intermediates of nitrate reduction are suggested to be toxic to

methanogens (Asanuma et al., 2014). Monensin reduces the number of H_2-producing bacteria (Chen and Wolin, 1979) and protozoal populations (Guan et al., 2006). Hence, nitrate and MON affect the H_2 pool in the rumen through different mechanisms. However, reduction in CH_4 output have to be obtained without disrupting other rumen functions such as digestion and production of VFA. As reported by Capelari and Powers (2017), when combined at higher doses, nitrate and MON also reduced IVDMD (average 9.2%) and increased nitrous oxide (**N_2O**) production (up to 75 times greater than control), which partially offset the benefit on CH_4 reduction. In addition, short-term batch culture trials do not allow observations on development of microbial resistance to additives, with special concern to MON (Guan et al., 2006). Hence, it is necessary to screen for dose combinations that more closely translates into effective, long-term reduction on CH_4 production without compromising productive parameters. Therefore, we hypothesized that encapsulated nitrate (**EN**) and MON additively reduce CH_4 production, and, if adaptation to MON occurs, EN would maintain CH_4 levels below that of control. The objective of this study was to determine the long-term effect of EN and MON, alone and in combination, on ruminal fermentation parameters.

3.3. Material and Methods

Experiments were conducted at Michigan State University and consisted of 2 studies one with 4 replications (**DAIRY**) and the other with 3 replications (**BEEF**), where a semi-continuous culture of mixed rumen microorganisms was used to assess the effect of EN, MON and EN+MON on rumen fermentation parameters (total gas production, CH_4 production, nitrous oxide (**N_2O**) production, VFA production, pH and nutrient disappearance) in 2 diets (DAIRY, 50:50 forage to concentrate; and BEEF, 15:85 forage:concentrate). Because MON resistance of microbial species have been reported to occur during the initial 4 wks following supplementation

(Guan et al., 2006; Carmean and Johnson, 1990; Johnson et al., 1997), each trial consisted of 37 d total, with the initial 7 d for stabilization of the system (steady-state) followed by 30 d of data collection. All procedures were consistent for each trial, unless otherwise stated. Animal procedures were approved and followed internal guidelines recommended by the Animal Care and Use Committee of Michigan State University (IACUC study number 11/15-175-00).

3.3.1. Experimental design and treatments

Trials were arranged as a completely randomized design in a 2 × 3 factorial arrangement of treatments. Nitrate (0, 1.25 and 2.5% of EN; GRASP Ind. & Com. LTDA EW|Nutrition GmbH, Curitiba, Brazil) and sodium monensin (0 and 4 mg/L; Elanco Animal Health, Greenfield, IN) were tested alone and in combination, totaling 6 treatments. The EN source was calcium ammonium nitrate decahydrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$; 85.6% DM, 17.6% N, 19.6% Ca, and 71.4% nitrate on a DM basis) designed to release 50, 80, and 100% of nitrate within 4, 12, and 30 h, respectively (El-Zaiat et al., 2014; Lee et al., 2015a,b; Lee et al., 2017). Urea and calcium carbonate were added when necessary to compensate for the additional non-protein nitrogen (**NPN**) and Ca provided with EN, therefore maintaining N and Ca levels similar across treatments. The fermentation system consisted of 18 vessels; each treatment replicated 3 times per trial. Vessel within trial was considered the experimental unit. The MON level tested (4 mg/L of culture media) was chosen because it represents an intermediary level of that tested previously, where the higher MON level caused negative responses to IVDMD when combined with the higher dose of nitrate (Capelari and Powers, 2017). The nitrate levels tested were chosen because 2.5% of DM was considered as a threshold for nitrate supplementation due to inhibition of feed intake (Lee et al., 2017) while the same level has shown to consistently reduce CH_4 levels, *in vivo* (Lee and Beachemin, 2014).

Two contrasting basal diets were used for the 7 trials. DAIRY trials (4 total) were conducted with a basal diet consisting of a 50:50 forage to concentrate ratio commonly fed to high producing dairy cows in the upper Midwest, and BEEF trials (3 total) used a high concentrate diet (15:85 forage to concentrate ratio; Table 1). Sufficient diet substrate for both DAIRY and BEEF trials were prepared in a single day in order to avoid changes in ingredients chemical composition. Following preparation as a total mixed ration (**TMR**), basal diets were dried for 48 h at 55 °C, ground through a 5-mm screen (Wiley Mill Model 4, Thomas Scientific, Swedesboro, NJ) and frozen at -20 °C until use. Prior to initiation of each trial, basal diets were thawed, basal diet aliquots (20 g) were weighed and additives (EN and MON), urea and calcium carbonate were added to basal diet as needed to obtain final treatment diets (Table 2). Treatment diets were stored in pre-labeled and pre-weighed nylon bags (10 × 20 cm; 50 μm mesh size; ANKOM Technology, Macedon, NY) until day of use.

3.3.2. Rumen simulation system

The rumen simulation system was designed and constructed at Washington State University Department of Animal Science Farm Shop and represented an adaptation of the original Rusitec system (Czerkawski and Breckenridge, 1977). The system had the capacity to administer artificial saliva, collect overflow and gas collection and accommodate feeding of different diets. The equipment was similar to that described by Czerkawski and Breckenridge (1977), but it contained 18, 2.2- L vessels and located the artificial saliva inflow on top of vessels. Vessels were collectively located in a large water bath (approximately 400 L volume) with 2 immersion-circulating heaters that maintained the temperature at 39 ± 0.5 °C. Vessels were gas-tight with a cap that, when fixed, maintained the fermentation vessels thermodynamically as a closed system. Located on the top of caps was an inlet for artificial

saliva, an outlet for the gas that accumulated in the headspace, and an outlet for effluent. The artificial saliva reservoir was connected to vessels through tygon tubing (3.1 mm ID × 6.3 mm ED; Cole-Parmer Lab Supplies, Vernon Hills, IL) connected to a peristaltic pump that was installed at the top of the apparatus. In order to store the gas for a 24 h period, gas outlets were connected to 10-L Tedlar sampling bags (Supelco Inc, Bellefonte, PA). Liquid effluent was stored in 2-L plastic containers connected to each vessel by tygon tubing (9.5 mm ID × 12.7 mm ED; Cole-Parmer Lab Supplies, Vernon Hills, IL).

A hydraulic pump was used to simulate rumen movements. A perforated feed container containing diet bags with treatment was located in each vessel and connected to the hydraulic pump in order to mix vessel contents, avoiding the formation of dead spaces, and allowing contact between liquid rumen fluid and feed bags.

3.3.3. Incubation conditions and initial experimental procedures

A mixture (1:1; 1.2 L) of artificial saliva (Mc Dougall, 1948; pH = 8.3; 9.8 g/L of $NaHCO_3$, 3.72 g/L of Na_2HPO_4, 0.47 g/L of NaCl, 0.57 g/L of KCl, 0.053 g/L of $CaCl_2 \cdot 2H_2O$, 0.128 g/L of $MgCl_2 \cdot 6H_2O$ and 0.3 g/L of $(NH_4)_2SO_4$) and water was added to all vessels on D -8. Ruminal inoculum donors were housed at the Michigan State University Dairy Cattle Teaching and Research Facility. On D -7 rumimal inoculum was collected from 5 ruminally fistulated lactating cows (different cows in each trial) consuming a 50:50 roughage: concentrate diet similar to that used for DAIRY trials (DM basis - approximately 18% ground corn, 18% alfalfa haylage, 15% soybean meal, 15% high moisture corn and 18% corn silage) in the form of TMR. Diets of rumen fluid donors did not contain any ionophore or supplementary nitrate. Rumen fluid was manually collected 3 h following morning feeding from 4 different rumen locations of each donor and transferred to 3 pre-heated 30-L insulated containers. The ruminal inoculum was

immediately transported to Michigan State University Animal Air Quality Research Facility where it was blended and filtered through 2 layers of cheesecloth. The processed fluid (1 L) was transferred under continuous CO_2 flow to each vessel. Approximately 40 g of wet solid rumen contents from inoculum donors were weighed into a pre-labeled bag and added to feed containers on D -7 along with 20 g of basal diet. Following incubation with rumen inoculum, vessels were closed and the peristaltic pump and hydraulic pump were started. Artificial saliva was prepared daily with a dilution rate set to 0.7 d^{-1} (1600 mL/d) and hydraulic pump set for 3 complete rotational movements per minute. Bags containing solid rumen contents were removed from feed containers on D -6 and replaced with a second bag of basal diet. From D -6 through D 0, 2 bags with 20 g of basal diet was present in each vessel for 48 h. Each morning at 0600 h a new feed bag was added to all vessels and the bag that had been in the vessel 48 h was removed. On D 0 the feed bag added contained the assigned experimental treatment.

3.3.4. Sampling and analytical procedures

Daily, before feed bag exchange, effluent volume was measured and bags containing gas produced over 24 h were closed and replaced by a second set of bags. Following, all vessels were opened (3 at a time), flushed with pure CO_2 to maintain anaerobic conditions, and a 5 mL sample was taken from liquid phase for VFA analysis. After a new feed bag was added to feed containers, vessels were immediately closed and CO_2 was flushed in excess for 5 s through the gas outlet port. Gas samples were collected from gas bags with a 10 mL syringe (Model 1010 C, Hamilton Company, Reno, NV) and transferred to 20 mL gas chromatography (**GC**) vials containing 15 mL of ultra-high purity N_2 gas for off-site analysis for CH_4, N_2O and CO_2 concentrations. The analytical procedures used for gas and VFA considered concentration of analytes obtained by GC and gas and effluent volume and it was described in detail previously

(Capelari and Powers, 2017). Total gas production was calculated by difference between relative pressure, corrected for temperature and humidity, and measured using a relative pressure gauge (Model Media Gauge, SSI Technologies, Jonesville, WI). A homemade container system (5 L) enabled the transfer and measurement of gas samples accumulated in the bags. To determine gas volume by pressure difference the container was connected to a vacuum pump and the pressure gauge. The vacuum pump was started and when the pressure reading stabilized (approximately 3.44 kPa) a gas valve was closed and the value noted. Each bag was then connected and the container was opened with the gas content of bags transferred via negative pressure to the container. The relative pressure of container was measured and recorded for a second time and the value used in the equation as follows:

$$Vgas = (V_c / P_{EL}) * P_{BT} \quad [1]$$

where Vgas is the volume of gas production, ml; V_c is the volume of solid container, 5000 ml; P_{EL} is the atmospheric pressure in East Lansing, MI, 101.3 kPa; and P_{BT} are the pressure measurements from the gauge, psi (final read – initial read).

Samples for analysis of NH_3-N, nitrate and nitrite (**NO_2**) in rumen fluid were collected on D 0, 2, 9, 16, 23 and 30. For NH_3-N, an aliquot of 30 mL was sampled directly from the vessels before feed bag exchange and transferred to 50 mL tubes containing 1 mL of concentrated sulfuric acid. The samples were frozen at – 20 °C until analysis of NH_3-N using a Kjeldahl distillation apparatus (KJELTEC system 1002 distilling unit, FOSS, Eden Prairie, MN). Samples for nitrate and NO_2 analysis (5 mL) were centrifuged (15,000 × g for 15 min at 4°C) and concentration was measured with a colorimetric assay kit (Cayman Chemical Company, Ann

Arbor, MI). On the same days, pH of the fermentation vessels was measured using a pH meter (Model HQ40d Portable pH meter, HACH Co, Reno, NV).

Nutrient disappearance over 48 h was measured twice weekly on D -1, 1, 2, 8, 10, 15, 17, 22, 24, 28 and 29. Feed bags withdrawn from the vessels were gently washed with water until the effluent ran clear. The bags were dried for 48 h at 55 °C for DMD determination. The residues from the 11 sampling d were then pooled, equally, and ground through a 1 mm screen (A.H. Thomas, Philadelphia, PA, USA). Samples were further dried at 105°C for 2 h. Composite samples from residues were then analyzed for DM, NDF, starch and CP. Chemical analyses were performed on each sample, in triplicate, and when the coefficient of variation was greater than 5% the analysis was repeated. The NDF was determined according to Van Soest et al. (1991) with heat-stable amylase and sodium sulfite used in the procedure. Starch content was determined by enzymatic hydrolysis of α-linked glucose polymers as described by Karkalas (1985). Total Kjeldahl N (TKN) content was analyzed according to standard method (AOAC, 1990), and CP was calculated as N × 6.25. Samples of ingredients used in the total mixed ration in DAIRY and BEEF trials were analyzed for DM, CP, NDF, nonfiber carbohydrates, starch, and ash by wet chemistry (Dairy One Forage Analysis Laboratory, Ithaca, NY).

3.3.5. Fermentation balance calculations

All equations used here are described in detail by Wolin (1960), Demeyer and Tamminga (1987) and Demeyer (1991). In summary, the ratio of H_2 utilized and H_2 produced (μ moles/ mL; Eq. 2 and 3, respectively) is used to calculate percent H_2 recovery (%; Eq.4). The H_2 produced as fermentation end products and H_2 consumed to form CH_4 and VFA were determined from molar concentration of acetate (C_2), propionate (C_3), butyrate (C_4), isovalerate (Ci_5), valerate (C_5) and

CH_4. The equations do not account for H_2 released in the gaseous form, lactate, microbial mass, and potential acetate produced via reductive acetogenesis.

$$H_2 \text{ utilized} = (2 \times C_3) + (2 \times C_4) + (4 \times CH_4) + Ci_5, \qquad [2]$$

in which H_2 utilized is expressed as micromoles per milliliters,

$$H_2 \text{ produced} = (2 \times C_2) + C_3 + (4 \times C_4) + (2 \times C_5) + (2 \times Ci_5), \qquad [3]$$

in which H_2 produced is expressed as micromoles per milliliters,

$$H_2 \text{ recovery} = (H_2 \text{ utilized}/ H_2 \text{ produced}) \times 100, \qquad [4]$$

in which H_2 recovery is expressed as micromoles per milliliters,

The amount of hexose (C_6) fermented was calculated as follows:

$$\text{hexose fermented} = (0.5 \times C_2) + (0.5 \times C_3) + C_4 + C_5, \qquad [5]$$

in which hexose fermented is expressed in micromoles per milliliter.

Fermentation efficiency was calculated by considering the heat of combustion of glucose, acetate, propionate and butyrate and their molar concentration (mM), as follows:

$$\text{fermentation efficiency} = \{[0.62 \times C_2) + (1.09 \times C_3) + (0.78 \times C_4)]/(C_2 + C_3 + C_4)\} \times 100, \qquad [6]$$

in which fermentation efficiency is expressed as a percent.

3.3.6. Statistical analysis

Data were tested for normality and submitted to analyses of variance with 5% significance level. The MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) was used for all statistical

analysis and inferences. Means for all variables were obtained by LSMEANS. The statistical model was as follows:

$$Y_{i(k)j\mathrm{m}} = \mu + \beta i_{(k)} + \tau_{\mathrm{j}} + \lambda_{\mathrm{m}} + e_{i(k)j\mathrm{m}}$$

where $Y_{ij(k)m}$ represents observation $_{ij(k)m}$; μ represents the overall mean; β_i represents the random effect of *i*th vessel within *k*th trial; τ_j represents the fixed effect *j*th treatment and λ_m represents the fixed effect of the *mth* day. The residual terms $\varepsilon_{ij(k)m}$ were assumed to be normally, independently, and identically distributed with variance σ^2_e. The REPEATED statement was used for variables measured daily (gas variables and VFA profile). Tukey correction was used to separate treatment means and a level of $P \leq 0.05$ was used to determine significance, and tendencies were associated with P-values between 0.05 and 0.10.

3.4. Results

3.4.1. DAIRY trials (50:50 roughage: concentrate)

The addition of EN reduced total daily gas production when compared to control (2.54 vs. 2.47 L/d; $P < 0.01$; Table 3.3). When compared to control, EN and MON significantly reduced CH_4 production (24.77 and 26.34 vs. 32.15 mM/d, respectively; $P < 0.01$). The combination of EN + MON further reduced CH_4 production when compared to control (21.34 vs. 32.15 mM/d; $P < 0.01$). There was no effect of treatments on CO_2 production, however N_2O was increased by EN (41 vs. 33 ($\times 10^{-4}$) mM/d; $P < 0.01$). There were no effect of treatments on DM and NDF disappearance, however, EN reduced CP (96.9 vs. 97.6 %; $P < 0.01$) and starch (79.0 vs. 80.8 %; $P < 0.01$) disappearance.

Compared to control, total VFA production was increased by MON (38.3 vs. 35.4 mM; $P < 0.01$; Table 3.4). The primary effect of monensin of VFA molar proportions was a reduction in

acetate proportion (47.6 vs. 49.9 mol/100 mol; P < 0.01) and an increase in propionate proportion (26.8 vs. 22.2 mol/ 100 mol; *P* < 0.01). As a consequence the A:P ratio was reduced by MON when compared to control (1.6 vs. 2.0; P < 0.01) while EN tended to increase A:P when compared to control (2.15 vs. 2.0; *P* < 0.1) despite no effect on acetate and propionate individually. However, butyrate was reduced by MON (16.1 vs 19.4 mol/ 100 mol; *P*< 0.01), EN (18.2 vs. 19.4 mol/ 100 mol; *P* < 0.05) and with further reduction by EN+MON (15.2 vs. 19.4 mol/ 100 mol; *P* < 0.1). Nitrate significantly reduced NH_3-N concentration (17.1 vs. 21.09 mg/dL; *P* < 0.01) and increased both nitrate and nitrite (*P* < 0.01) in rumen fluid. No effect of treatments was observed for pH (6.8 ± 0.3).

3.4.2. BEEF trials (15:85 roughage: concentrate)

Total gas production when compared to control was reduced by EN (3.05 vs. 3.25 L; *P* < 0.01), MON (3.06 vs. 3.25 L; *P* < 0.01) and with further reduction by EN + MON (2.66 vs. 3.25; *P* = 0.06; Table 5). When compared to control, CH_4 production was decreased by EN (33.9 vs. 43.5 mM/d; *P* < 0.01), MON (26.7 vs. 43.5 mM/d; *P* < 0.01) and further reduced when additives were combined (19.3 vs. 43.5 mM/d; *P* = 0.01). The EN treatment decreased CO_2 production when compared to control (98.6 vs. 103.0 mM/d; *P* < 0.01), but no treatment effect was observed on N_2O production. Feeding EN significantly reduced disappearance of DM (73.5 vs. 74.4%; *P* < 0.01), NDF (29.3 vs. 32.5%; *P* < 0.01) and starch (88.5 vs. 90.35 %; *P* < 0.01) while MON significantly reduced disappearance of NDF (29.34 vs. 32.5%; *P* < 0.01) and increased CP (83.9 vs. 82.5%; *P* < 0.01).

Total VFA production increased by MON (50.7 vs. 48.1; *P* < 0.01) and by EN+MON (52.3 vs. 48.1; Table 6; *P* < 0.01). The molar proportion of acetate tended to increase due to EN+MON (51.2 vs. 49.5 mol/ 100mol; *P* = 0.07) while the propionate molar proportion was increased by

MON (29.6 vs. 26.2 mol/100 mol; $P < 0.01$). Feeding monensin reduced the butyrate molar ratio when compared to control (12.6 vs. 14.9 mol/100 mol; $P < 0.01$) and isovalerate (1.6 vs. 2.0 mol/100 mol; $P < 0.01$). Because MON increased propionate the A:P ratio was reduced by MON when compared to control (1.6 vs. 1.9; $P < 0.01$). Nitrate significantly reduced NH_3-N concentration (6.63 vs. 8.14 mg/dL; $P < 0.01$) but increased both nitrate and nitrite ($P < 0.01$) in rumen fluid. No effect of treatments was observed for pH (6.7 ± 0.3).

3.5. Discussion

3.5.1. Fermentation products

The rumen simulation technique (Rusitec) was developed for testing of fibrous feed (Czerkawski and Breckenridge, 1977), but the approach has been successfully used for mixed diets with different amounts of starch (Carro et al., 1992; Carro et al., 2009), for evaluation of feed additives on rumen metabolism (McAllister et al., 1994), and for development of CH_4 mitigation strategies (Avilla-Stagno et al., 2014; Romero-Pérez et al., 2015). The Rusitec presents several positive features for rumen studies, such as 1) sampling of fermentation products, like gas and ruminal fluid, 2) testing of different treatments, with sufficient replication, and the 3) use of high concentrations of feed additives, which could potentially be toxic to animals, all at a 4) lower cost compared to animal trials. However, caution should be taken when interpreting data and trying to extrapolate results obtained from rumen simulation techniques to *in vivo* situations due to microbial community differences, lack of absorption of fermentation end products and low substrate to rumen volume ratio (Mansfield et al., 1995; Hristov et al., 2012).

In the current study, on average, EN reduced CH_4 production by 24 and 22% when compared to control, for the DAIRY and BEEF diets, respectively, confirming the potential of EN as a CH_4 mitigation option for ruminants. For DAIRY, mitigation efficacy of EN (% CH_4

reduction per 1% nitrate in the diet, assuming full reduction to NH_3) was 13.4 and 11.7% for 1.25 and 2.5% of EN in diet DM, respectively, while efficacies in BEEF were 14.5 and 10.4%. These results are similar to observed by others and demonstrate a reduction in efficacy as nitrate inclusion increases regardless of diet composition (12.2%, Van Zijderveld et al., 2010; 12.5% Lund et al., 2014; 7.3% Lee et al., 2015a; 9.2%, Veneman et al., 2015; 11.4%, Guyader et al., 2017). When the antimethanogenic effect of nitrate was tested in batch culture trials at 1.25 and 2.5% of diet DM, CH_4 production was reduced by an average 62.5% (for two contrasting diet substrates) which is above the expected when animal models are used, but likely reflect a limitation of closed systems to maintain rumen conditions overtime (Capelari and Powers, 2017). When evaluating the dose-effect of nitrate on CH_4 production of Holstein steers, Newbold et al. (2014) observed efficiencies ranging from 19.5% when nitrate level was 0.6% of diet DM to 9.6% when nitrate was added at 3%. The decrease in efficacy as nitrate inclusion increases can be explained by an incomplete reduction of nitrate to NH_3 and by the divergence of H_2 from propionogenesis and microbial cell synthesis rather than from methanogenesis. In animals, the absorption of nitrate and NO_2 into the bloodstream before complete reduction to NH_3 could also explain reduced efficacy as discussed by Nolan et al. (2016). However, efficacy of CH_4 mitigation can increase if nitrate intermediates reduce the number of H_2 generating microorganisms or the number of methanogens (Kluber and Conrad, 1998).

In the rumen, nitrate is reduced to NH_3 and for every mole of nitrate reduced CH_4 production should decrease by 1 mole. Stoichiometrically, the complete reduction of nitrate to NH_3 consumes 4 moles of H_2, the same number of H_2 molecules necessary for methanogens to reduce CO_2 to CH_4. Thermodynamically, both reactions involved in nitrate reduction to NH_3 are energetically favorable compared to reduction of CO_2 by methanogens (Ungerfeld and Kohn,

2006). Further, intermediates of nitrate reduction such as nitrite (**NO_2**) are suggested to have a toxic effect on methanogens (Lee and Beauchemin, 2014), therefore at least part of the CH_4 decrease observed *in vitro* (Marais et al., 1988; Guyader et al., 2017; Capelari and Powers, 2017) and *in vivo* (Newbold et al., 2014; Lee et al., 2015a; Guyader et al., 2015) have been linked to NO_2 effect on methanogenic archaea population. When Capelari and Powers (2017) tested 3 nitrate levels in short-term batch culture trials, the inhibition of CH_4 due to nitrate effect as H_2 sink explained, on average, 67.5% of the observed CH_4 decrease (actual CH_4 mitigation in moles / theoretical mitigation potential, assuming complete nitrate reduction to NH_3). In addition, N_2O accumulated in the headspace only when nitrate was present, suggesting an incomplete reduction of nitrate to NH_3 and supporting the hypothesis that nitrate can affect methanogenesis through a secondary mechanism. Using the same calculations in the current experiment, the effect of EN as a H_2 sink would explain, on average, 79 and 61% of actual CH_4 reduction observed in DAIRY and BEEF trials, respectively. Increased NO_2 levels in rumen fluid were observed when EN was fed, regardless of diet. Because samples for nitrate and NO_2 concentration analysis were collected approximately 24 h after feeding (before feed bag exchange), we can speculate higher values occurred immediately after feeding. Lee et al. (2015a) observed a significant increase in rumen NO_2 levels 3 h after feeding EN to beef heifers, but did not detect any NO_2 left in the rumen fluid 6 h after feeding. Guyader et al. (2015) observed elevated rumen dissolved H_2 up to 2 h after feeding, which was suggested to be related to a toxic effect of nitrate or its intermediates on H_2 utilizers such as methanogens, and Asanuma et al. (2014) fed nitrate to goats (6 and 9 g/d) and observed a significant reduction in methanogen copy numbers as estimated by real-time PCR. Monensin does not affect major species of nitrate reducers in the rumen, such as *Selenomonas ruminatium* (Chen and Wolin, 1979) and *Veillonella parvula* (Newbold et al.,

1993). However, the combination of EN + MON numerically increased NO_2 levels in rumen fluid in DAIRY and tended to increase NO_2 in BEEF trials, suggesting a possible influence of MON on nitrate reduction in the rumen. Studies in plants have reported that nitrate transport is powered by electrochemical potential of protons across the plasma membrane (Ullrich, 1992) with high and low affinity transport systems demonstrating saturation kinetics (Wang and Crawford, 1996; Crawford and Glass, 1998). Monensin reduced the nitrate induced depolarization in plant leaf cells by 85% (Garcia-Sanchez et al., 2000). An effect of MON on nitrate reduction in the rumen would be undesirable and deserves further investigation because accumulation of NO_2 in the rumen is related to reduced diet digestibility (Marais et al., 1988) and methemoglobinemia (Lee and Beauchemin, 2014). Research efforts have targeted reduced NO_2 accumulation in the rumen (Nolan et al., 2016; Yang et al., 2016).

As an ionophore, MON has the ability to form lipid-soluble complexes with cations and mediate their transport across lipid barrier of gram positive bacteria (Russell and Strobel, 1988). Further, MON also inhibits the growth of fungi (Steward et al., 1987; Steward and Richardson, 1989) and transiently inhibits the growth of ciliate protozoa (Hino, 1981; Wallace et al., 1981; Guan et al., 2006; Sylvester et al., 2009). The result is a decrease in CH_4 production (Odongo et al., 2007) resulting from an increase in propionate production to the detriment of acetate and butyrate production (Guan et al., 2006), and a protein-sparing mechanism, increasing availability of true protein (Van Nevel and Demeyer, 1977). The present study observed that MON decreased CH_4 output by 19.8 and 39.6% for DAIRY and BEEF trials, respectively. This is consistent with previous *in vitro* and *in vivo* studies (Van Nevel and Demeyer 1977; Thornton and Owens 1981; Wallace et al. 1981; Wedegaertner and Johnson 1983; Martin and Macy, 1985; Dong et al., 1999; Jenkins et al., 2003; Capelari and Powers, 2017). The increase in rumen

propionate is accompanied by a decrease in the amount of CH_4 production because MON reduces the population of H_2 generating bacteria (Van Nevel and Demeyer, 1977; Wallace et al.,1981), which is supported by the inability of MON to reduce CH_4 production when H_2 and CO_2 are provided (Russell and Martin, 1984). Moreover, during one of the rumen pathways for propionate formation, fumarate is reduced to succinate and 1 mole of H_2 is consumed, resulting in a net uptake of H_2 (Ungerfeld and Kohn, 2006). No sign of microbial resistance was detected indirectly through the variables analyzed during the current study (data not shown), and CH_4 decrease resulting from MON addition was maintained until D 30. An observed additive effect of EN+MON on CH_4 production suggests that additives that alter the H_2 pool in the rumen through different mechanisms might further reduce CH_4 output, such as that observed when nitrate was combined with lipids (Guyander et al., 2015), sulfate (Van Zijderveld et al., 2010) and saponin (Patra and Yu, 2014). The additive effect of nitrate and MON on reducing CH_4 production when compared to additives alone also agrees with findings from Capelari and Powers (2017) who tested 3 levels of nitrate and MON in short-term batch culture trials, which suggests that the strategy is effective in terms of CH_4 abatement.

Consistent across the DAIRY and BEEF trials, the addition of EN significantly reduced NH_3 concentration in fermentation media in a dose-response manner although diets were iso-nitrogenous. The effect has been reported in other *in vitro* (Lin et al., 2011; Guyander et al., 2017) and *in vivo* (El-Zaiat et al., 2014; Lee et al., 2015a) trials. Guyander et al. (2017), in an attempt to track the fate of H_2 when CH_4 was inhibited with different feed additives, reported concomitant reduction in NH_3 and increased microbial protein synthesis in Rusitec fermenters fed EN. Increased efficiency of microbial protein synthesis could explain the reduced NH_3 observed in the current study as more NH_3 would be incorporated into microbial biomass (Nocek

and Russell, 1988). More studies measuring microbial protein synthesis in animals fed nitrate could clarify the mechanism for the observed effect of nitrate on rumen N metabolism. Alternatively, the form of nitrate fed could influence the NH_3 concentration. The nitrate form used in the trials was encapsulated, where nitrate is protected by a matrix that regulates the release rate in rumen fluid (Lee et al., 2017). During the experiment it was possible to visually identify and count intact capsules after 48 h of incubation (approximately 5% of total number of EN capsules initially added; data not shown). Because treatments not receiving EN were iso-nitrogenous through addition of urea (not encapsulated) part of the effect on NH_3 concentration could be explained by incomplete release of nitrate content. Finally, reduction of nitrate to NH_3 is a slower process than the immediate conversion of urea to NH_3 in the rumen, which is confirmed by the significant levels of nitrate and NO_2 observed in rumen fluid 24 h post feeding EN.

3.5.2. Fermentation balance

Stoichiometric calculations are helpful in describing alterations in fermentation, but they must be interpreted with caution because manipulating fermentation may invoke reactions not considered in the calculations and fermentation uncoupled from cell growth may occur (Chalupa, 1977). Reductions in metabolic H_2 production were observed when MON was added to both DAIRY and BEEF feeds as a consequence of reduced acetate and butyrate concentrations in support of increased propionate. Acetate and butyrate formation releases 4 moles of H_2 per mole of glucose fermented. Propionate formation, on the other hand, requires a net input of H_2 (Ungerfeld and Kohn, 2006). Methane is the primary H_2 sink in the rumen, and as consequence of reduced CH_4 formation when additives were present, utilization and recovery of metabolic H_2 was significantly decreased by the treatments. Even though gaseous H_2 was not measured, it is

predicted that a part of the H_2 spared from methanogenesis when EN was fed exit the rumen as gaseous H_2 as previously documented *in vivo* (Van Zijderveld et al., 2011; Guyader et al., 2015; Olijhoek et al., 2015) and *in vitro* (Guyader et al., 2017).

In rumen studies, fermentation efficiency can be defined as a measure of the energy present in a fermentable substrate (i.e., hexoses and AA) that is recovered in usable forms, such as VFA and microbial protein, to the host animal. From an energy stand point, production of propionate from hexose fermentation is more efficient than production of other VFA because less energy is lost as H_2 and C. Therefore, shifts in rumen fermentation caused when MON in both DAIRY and BEEF diets were fed resulted in significant increases in overall fermentation efficiency due to greater propionate concentration. However, it should be stressed that the profile of VFA produced in the rumen also has consequences on the host animal metabolism. Excess propionate can lead to undesirable consequences such as reduction in voluntary feed intake (Oba and Allen, 2003; Allen et al., 2009) and low milk fat content and fat syndrome in lambs (Orskov and Ryle, 1990).

3.5.3. Nutrient digestion

The disappearance of DM and other chemical fractions for both the DAIRY and BEEF trials were within expected values for relatively good quality diet substrates and were similar to those observed by Dong et al. (1999) and Avila-Stagno et al. (2014) when testing the effect of additives and different levels of glycerol in fermenters fed high quality hay and corn silage-based diets. Nitrate reduced CP and starch disappearance in DAIRY trials and NDF and starch fractions in BEEF trials. Results suggest that nitrate or a reduction product temporarily present in the media can affect utilization of some chemical components of the diets (through solubilization

in ruminal fluid and/or degradation by microorganisms enzymes). Maraes et al. (1988) reported suppression of *in vitro* digestion of diet DM when NO_2 was present, but not when nitrate was added, thereby proposing that NO_2 was the primary factor affecting digestibility in the rumen. In the same study, NO_2 was shown to reduce solubilization of structural components of the diet, such as cellulose and hemicellulose, confirmed by a reduction in the cellulolytic and xylanolytic microbial populations (*Ruminococcus Flavefaciens*, *Ruminococcus albus* and *Butyrivibrio Fibrisolvens*) with concomitant reduction in cellulase and xylanase activity. Reduction of ADF digestibility has been reported in beef heifers fed increasing levels of nitrate on a barley silage and ground corn-based diet despite an increase in total-tract DM digestibility, likely due to increased digestibility of starch (Lee et al., 2015a). Asanuma et al. (2014) reported increased starch-utilizing organisms when goats were fed 9 g/d nitrate. Encapsulation of nitrate, especially in BEEF diets, may limit the synchronization between readily available carbohydrates (NFC in DAIRY = 46.6 and BEEF = 64.4 % of diet DM; Table 1) and N sources to rumen starch utilizers, therefore reducing the growth of these microorganisms. In addition, the Rusitec is a limited system in terms of hosting the complete rumen microbiome (Hristov et al., 2012; Martínez-Fernández et al., 2015) thus preventing the observation of potential benefits on specific groups of microorganisms. These results are in contrast to previous findings by Capelari and Powers (2017) who did not observed reduction to IVDMD when nitrate was added to batch cultures. Still, the observed reduction in digestion of chemical components when EN was present demonstrate that more animal studies are needed to better understand interactions among animal, diet and microorganisms and avoid detrimental effects of supplementary nitrate to animal performance.

The effect of MON on nutrient digestibility has been inconsistent in both *in vitro* and *in vivo* studies. In the current study, MON did not affect nutrient and DM disappearance in DAIRY trials but reduced NDF disappearance in BEEF trials by 10% when compared to control. Wallace et al. (1981) reported a reduction in cellulose and hemicellulose digestion when the Rusitec was fed 10 mg/L of MON daily, a dose more than 2 times that tested in the current trials. Dong et al. (1999) found a reduction in cellulose (30%) and hemicellulose (21%) digestion due to MON when the Rusitec received 20.5 µM/d of MON. In various *in vitro* and *in vivo* studies, MON has depressed fiber digestion (Poos et al. 1979; Mir, 1989), affected fiber digestion minimally (Ricke et al. 1984; Faulkner et al. 1985) or not at all (Kone and Galyean, 1990; Duff et al.1995), and even to increased fiber digestibility (Wedegaertner and Johnson, 1983). Potential negative effects of MON on ruminal fiber degradation are likely related to the sensitivity of the 3 primary cellulolytic species, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*, to MON (Chen and Wolin, 1979). Factors such as the dosage used, diet chemical composition and retention time of digesta may account for variability in results.

3.6. Conclusion

In summary, the combination of EN and MON can serve as a potential mitigation strategy for enteric CH_4 abatement, in agreement with previous findings from Capelari and Powers (2017). However, caution should be taken with dosage because the digestion of feed components can be impaired by additives, especially when they are combined at higher dosages. Animal studies to evaluate the combination of nitrate and MON on rumen and metabolic parameters as well as animal performance and health are needed before recommending this dietary strategy under field conditions.

Table 3.1. Ingredients and chemical composition of basal diets for DAIRY (50:50 roughage: concentrate) and BEEF (15:85 roughage: concentrate) trials

Ca	DAIRY	BEEF
Ground corn	18.1	
Alfalfa haylage	17.9	
Soybean meal	15.8	
High moisture corn	15.7	73.0
Corn silage	17.9	15.0
Dry distillers		8.0
Cottonseeds	6.5	
Wheat straw	2.7	
By pass fat[1]	2.0	
Premix[2]	2.0	4.0
Sodium bicarbonate	0.75	
Calcium carbonate[3]	0.66	
Analyzed chemical composition (% of DM)		
DM	56.4	64.0
CP	17.5	12.1
NDF	25.0	15.4
NFC	46.6	64.4
Starch	29.2	57.5
Ash	5.5	3.9

[1]Megalac (97% DM; 6.83 Mcal/kg DM; 84.5% fat; 15.5% ash).

[2]Premix contained 17.0% Ca, 8.0% P, 10.0% Mg, 3.000 mg/Kg Fe, 75 mg/Kg I, 8.000 mg/Kg Zn, 2.500 mg/Kg Cu, 2.000 mg/Kg Mn, 45 mg/Kg Co, 2.000 mg/Kg F, 1.666.000 IU/Kg Vit. A, 366.666 IU/Kg Vit. D and 20.000 IU/Kg Vit.E.

[3]Calcium carbonate contained 38% Ca.

Table 3.2. Composition of experimental treatments of the Rusitec DAIRY (50:50 roughage: concentrate) and BEEF (15:85 roughage: concentrate) trials[1]

Item, DM, % (unless otherwise stated)	0 EN		1.25 EN		2.5 EN	
	0 MON	4 MON	0 MON	4 MON	0 MON	4 MON
Basal diet	97.5	97.5	97.5	97.5	97.5	97.5
Nitrate source[2]	0	0	1.25	1.25	2.5	2.5
Monensin[3], mg/L	0	4	0	4	0	4
Urea	1.1	1.1	0.6	0.6	0	0
Calcium carbonate[4]	1.3	1.3	0.6	0.6	0	0

[1]EN = encapsulated calcium nitrate (0, 1.25 and 2.5% of diet DM); MON = sodium monensin (0 and 4 mg/L).

[2]Nitrate source was manufactured by GRASP Ind. & Com. LTDA and EW|Nutrition GmbH. The source of nitrate was the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$]; 85.6% DM; 17.6% N, 19.6% Ca, and 71.4% nitrate on a DM basis.

[3]Monensin sodium ($C_{36}H_{61}NaO_1$) was manufactured by Elanco Animal Health as Rumensin 90; 198g of active ingredient per kg.

[4]Calcium carbonate contained 38% Ca.

Table 3.3. Effects of EN and MON on gas production and nutrient disappearance on DAIRY (50:50 roughage: concentrate) trials[1]

	0 EN		1.25 EN		2.5 EN			*P*-value[1]		
Item	0 MON	4 MON	0 MON	4 MON	0 MON	4 MON	SEM	EN	MON	EN×MON
Dilution rate (mL, d^{-1})		1526	1576	1545	1518	1514	27.91	NS	NS	NS
Gas production										
Total gas (L)	2.57[b]	2.57[b]	2.48[b]	2.60[b]	2.71[a]	2.40[c]	0.20	0.01	NS	NS
CH_4, mM/d	32.15[a]	26.34[b]	26.77[b]	23.66[c]	22.78[c]	19.03[d]	1.79	<0.01	<0.01	0.03
CO_2, mM/d	75.32	71.64	68.11	75.91	71.58	75.85	5.06	NS	NS	NS
N_2O ($\times 10^{-4}$), mM/d	33[c]	37[b]	35[b]	36[b]	39[ab]	46[a]	3.8	<0.01	NS	NS
Nutrient disappearance (%)										
DM	64.90	65.35	65.16	64.66	64.29	64.21	1.36	NS	NS	NS
NDF	27.64	26.64	28.21	28.4	28.56	26.36	1.15	NS	NS	NS
CP			97.0[b]	97.17[ab]	96.85[b]	96.86[b]	0.38	<0.01	NS	NS
Starch	80.78[a]		79.92[ab]	80.12[a]	78.17[b]	78.22[b]	0.63	<0.01	NS	NS

[a-d] Within a row, means without a common superscript letter differ, $P < 0.05$.

[1] EN = Encapsulated calcium nitrate (0, 1.25 and 2.5% of diet DM); MON = Sodium monensin (0 and 4 mg/L).

[2] NS = non significant; ($P > 0.10$).

Table 3.4. Effects of ENP and MON on fermentation parameters on DAIRY (50:50 roughage: concentrate) trials[1]

	0 EN		1.25 EN		2.5 EN			*P*-value[2]		
Item	0MON	4 MON	0MON	4 MON	0 MON	4 MON	SEM	EN	MON	EN×MON
pH	6.90	6.89	6.95	6.79	6.92	6.89	0.22	NS	NS	NS
Total VFA, mM	35.4^{b}	38.3^{a}	34.3^{b}	36.7^{b}	34.8^{b}	37.8^{ab}	2.08	NS	<0.01	NS
VFA composition, mol/100 mol										
Acetate (A)	49.9^{b}	47.6^{a}	50.0^{b}	53.7^{c}	50.8^{bc}	49.8^{b}	1.17	NS	NS	<0.01
Propionate (P)	22.2^{b}	26.8^{a}	20.4^{b}	27.5^{a}	21.7^{b}	26.1^{a}	0.74	NS	0.01	NS
Butyrate	19.4^{a}	16.1^{b}	19.6^{a}	15.1^{b}	16.9^{b}	15.3^{b}	0.59	<0.05	<0.01	NS
Valerate	7.3	8.4	7.0	7.4	6.9	7.2	0.44	NS	NS	NS
Isobutyrate	2.3	2.6	2.7	4.5	2.9	2.6	0.56	NS	NS	NS
Isovalerate	4.1	3.8	3.9	4.5	3.6	3.4	0.47	NS	NS	NS
Caproate	3.6	3.1	3.8	3.2	3.5	2.8	0.32	NS	<0.05	NS
A:P	2.0^{b}	1.6^{c}	2.2^{a}	1.6^{c}	2.1^{ab}	1.8^{c}	0.06	<0.1	<0.01	NS
Metabolic hydrogen balance,[3] µmol/mL of fermentation media										
Produced	205.8^{a}	197.3^{ab}	208.4^{a}	193.5^{c}	199.3^{ab}	197.2^{ab}	2.9	NS	<0.01	NS
Utilized	178.5^{a}	154.1^{b}	151.9^{b}	140.6^{bc}	136.7^{c}	125.7^{c}	4.8	<0.01	<0.01	<0.01
Recovered, %	86.7^{a}	78.2^{a}	73.3^{a}	72.0^{a}	68.6^{b}	63.4^{b}	3.8	<0.01	<0.01	<0.01
Hexose fermented	32.0	35.3	32.5	31.4	36.3	32.0	1.9	NS	NS	NS
Fermentation	76.6^{bc}	79.2^{a}	76.2^{c}	77.7^{b}	75.8^{c}	77.8^{b}	1.2	NS	<0.01	NS
NH_3-N(mg/dL)	21.09^{a}	20.77^{a}	18.10^{b}	18.48^{b}	16.21^{c}	17.73^{b}	0.72	<0.01	NS	NS
NO_3^-, uM	0	0	0.07^{b}	1.1^{a}	1.4^{a}	1.0^{a}	0.002	<0.01	NS	NS
NO_2^-, uM	0	0	0.7^{b}	3.6^{a}	4.7^{a}	1.2^{b}	0.01	<0.01	NS	NS

[a-d] Within a row, means without a common superscript letter differ, $P < 0.05$.

[1] EN = Encapsulated calcium nitrate (0, 1.25 and 2.5% of diet DM); MON = Sodium monensin (0 and 4 mg/L).

[2] NS = non significant; ($P > 0.10$).

[3] Stoichiometric calculations were based on balances applied to rumen fermentation (Wolin, 1960; Demeyer and Tamminga, 1987; Demeyer, 1991).

Table 3.5. Effect of EN and MON on rumen fermentation parameters of BEEF (15:85 roughage: concentrate) trials[1]

	0 EN		1.25 EN		2.5 EN		SEM	*P*-value[2]		
Item	0 MON	4 MON	0 MON	4 MON	0 MON	4 MON		EN	MON	EN×MON
Dilution rate (mL, d^{-1})	1523	1559	1511	1498	1557	1552	35.5	NS	NS	NS
Gas production										
Total gas (L)	3.25^{a}	3.06^{ab}	3.20^{ab}	2.93^{bc}	2.90^{c}	2.39^{d}	0.20	<0.01	<0.01	0.06
CH_4, mM/d	43.57^{a}	26.67^{c}	35.67^{b}	20.03^{d}	32.20^{b}	18.56^{d}	1.79	<0.01	<0.01	0.01
CO_2, mM/d	103.03^{a}	101.34^{a}	108.8^{a}	98.73^{ab}	88.43^{b}	88.61^{b}	4.23	<0.01	NS	NS
N_2O (x10^{-4}),	34	21	22	32	30	31	3.8	NS	NS	NS
Nutrient disappearance										
DM	74.38^{a}	74.90^{a}	74.54^{a}	73.97^{ab}	72.61^{bc}	71.70^{c}	1.36	<0.01	NS	NS
NDF	32.48^{a}	29.34^{b}	29.88^{a}	25.84^{b}	28.87^{ab}	24.6^{b}	1.73	<0.01	<0.01	NS
CP	82.56	83.94	82.68	83.17	81.98	82.37	0.51	NS	0.08	NS
Starch	90.35^{a}	90.24^{a}	89.3^{ab}	89.4^{ab}	87.8^{b}	87.43^{b}	0.64	<0.01	NS	NS

[a - d] Within a row, means without a common superscript letter differ, $P < 0.05$.

[1] EN = Encapsulated calcium nitrate (0, 1.25 and 2.5% of diet DM); MON = Sodium monensin (0 and 4 mg/L).

[2] NS indicates $P > 0.1$

Table 3.6. Effects of EN and MON on fermentation parameters on BEEF (15:85 roughage: concentrate) trials[1]

Daily means	0 EN		1.25 EN		2.5 EN		SEM	*P*-value[2]		
	0 MON	4 MON	0 MON	4 MON	0 MON	4 MON		EN	MON	EN×MON
pH	6.7	6.8	6.7	6.9	6.8	6.7	0.22	NS	NS	NS
Total VFA, mM	48.1[bc]	50.7[b]	44.7[c]	55.4[a]	47.0[bc]	49.2[bc]	1.9	NS	<0.01	<0.01
VFA composition, mol/100 mol										
Acetate (A)	49.5 [ab]	49.9[ab]	49.4[ab]	51.1[a]	48.6[b]	51.3[a]	1.17	NS	NS	0.07
Propionate (P)	26.2[b]	29.6[a]	26.0[b]	26.5[b]	27.3[ab]	27.2[ab]	0.74	NS	<0.01	NS
Butyrate	14.9[b]	12.6[c]	16.1[a]	13.4[c]	15.3[ab]	12.7[c]	0.59	<0.01	<0.01	NS
Valerate	4.6[c]	5.6[ab]	5.1[bc]	5.7[a]	4.8[c]	5.4[ab]	0.19	NS	<0.01	NS
Isobutyrate	1.03[a]	0.97[ab]	0.89[ab]	0.88[ab]	1.04[a]	0.79[b]	0.12	NS	0.02	0.03
Isovalerate	2.01[a]	1.61[b]	1.74[ab]	1.82[ab]	1.98[a]	1.47[b]	0.47	NS	<0.01	NS
Caproate	1.6	1.2	1.0	1.2	1.2	1.4	0.32	NS	NS	NS
A:P	1.9[a]	1.6[b]	1.9[a]	1.9[a]	1.8[ab]	1.9[a]	0.06	NS	<0.01	NS
Metabolic hydrogen balance,[3] μmol/mL of fermentation media										
Produced	196.8[a]	187.6[b]	198.4[a]	195.6[a]	195.1[a]	192.4[ab]	1.8	NS	<0.01	NS
Utilized	177.5[a]	103.1[c]	144.8[b]	85.5[d]	132.3[b]	82.1[d]	10.3	<0.01	<0.01	<0.01
Recovered, %	90.1[a]	54.9[c]	72.9[b]	43.4[d]	67.8[b]	42.6[d]	3.7	<0.01	<0.01	<0.01
Hexose fermented	41.9	41.8	39.6	43.5	39.4	42.1	1.3	NS	NS	NS
Fermentation efficiency, %	79.9	80.6	79.6	80.3	78.5	81.4	0.9	NS	<0.01	NS
NH_3-N, (mg/dL)		7.42[a]	7.35[a]	7.30[a]	5.92[b]	4.76[b]	0.41	<0.01	NS	NS

Table 3.6 (cont'd)

NO_3^-, uM	0	0	0.01	0.06	0.02	0.05	0.006	<0.01	NS	NS
NO_2^-, uM	0	0	0.1	0.17	0.11	0.16	0.008	<0.01	NS	<0.08

[a- d] Within a row, means without a common superscript letter differ, $P < 0.05$.

[1] EN = Encapsulated calcium nitrate (0, 1.25 and 2.5% of diet DM); MON = Sodium monensin (0 and 4 mg/L).

[2] NS = non significant; ($P > 0.10$).

[3] Stoichiometric calculations were based on balances applied to rumen fermentation (Wolin, 1960; Demeyer and Tamminga, 1987; Demeyer, 1991).

LITERATURE CITED

LITERATURE CITED

Allen, M. S., B. J. Bradford, and M. Oba. 2009. Board-Invited Review: The hepatic oxidation theory of the control of feed intake and its application to ruminants. J. Anim. Sci. 87: 3317-3334.

Asanuma, N., S. Yokoyama, and T. Hino. 2014. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to *Selenomonas ruminantium* having the ability to reduce nitrate and nitrite. Anim. Sci. J. 86: 378–384.

Alisson M. J., and C. A. Reddy. 1984. Adaptations of gastrointestinal bacteria in response to changes in dietary oxalate and nitrate. In: Proceedings of the Third International Symposium on Microbial Ecology, Washington, DC. p. 248 – 256.

Association of Official Analytical Chemists International. 1990. Official Methods of Analysis 15th ed. AOAC, Arlington, VA.

Avila-Stagno, J., A. V. Chaves, G. O. Ribeiro Jr, E. M. Ungerfeld, and T. A. McAllister. 2014. Inclusion of glycerol in forage diets increases methane production in a rumen simulation technique system. Br. J. Nutr. 111:829–835.

Capelari, M., and W. Powers. 2017. The effect of nitrate and monensin on *in vitro* ruminal fermentation. J. Anim. Sci. 95: 95:5112–5123.

Carmean, B. R., and D. E. Johnson. 1990. Persistence of monensin-induced changes in methane emissions and ruminal protozoa numbers in cattle. J. Anim. Sci. 68 (Suppl.1):517. (Abstr.).

Chen, M., and M. J. Wolin. 1979. Effect of monensin and lasalocid- sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. 38:72–77.

Carro, M. D., P. Lebzien, and K. Rohr. 1992. Influence of yeast culture on the in vitro fermentation (Rusitec) of diets containing variable portions of concentrates. Anim. Feed Sci. Technol. 37:209–220.

Carro, M. D., M. J. Ranilla, A. I. Martín-García, and E. Molina-Alcaide. 2009. Comparison of microbial fermentation of high- and low-forage diets in rusitec, single-flow continuous-culture fermenters and sheep rumen. Animal. 3:527–534.

Chalupa, W. 1977. Manipulating rumen fermentation. J. Anim. Sci. 45:585–599.

Crawford, N. M., and D. M. Glass. 1998. Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci. 3:389–395.

Czerkawski, J. W. and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). Br. J. Nutr. 38:371–384.

Demeyer, D. I. 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut. In: J. P. Jouany, editor, Rumen Microbial Metabolism and Ruminant Digestion. INRA Editions, Paris. p. 217–237.

Demeyer, D. I., and S. Tamminga. 1987. Microbial protein yield and its prediction. In: R. Jarrige and G. Alderman, editors, Feed Evaluation and Protein Requirement Systems for Ruminants. Office for Official Publications of the European Communities, Luxembourg City, Luxembourg. P. 129 – 141.

Dong, Y., H. D. Bae, T. A. McAllister, G. W. Mathison, and K. -J Cheng. 1999. Effects of exogenous fibrolytic enzymes, α-bromoethanesulfonate and monensin on fermentation in a rumen simulation (RUSITEC) system. Can. J. Anim. Sci. 79: 491-498.

Duff, G. C., M. I. Galyean, and M. E. Branine. 1995. Effects of adaptation to lasalocid, monensin or a daily rotation of lasalocid and monensin on in vitro fermentation of a 90% concentrate diet. Can. J. Anim. Sci. 75:129–134.

El-Zaiat, H. M., R. C. Araujo, Y. A. Soltan, A. S. Morsy, H. Louvandini, A. V. Pires, H. O. Patino, P. S. Correa, and A. L. Abdalla. 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. J. Anim. Sci. 92:2214–2224.

Faulkner, D. B., T. J. Klopfenstein, T. N. Trotter, and R. A. Britton. 1985. Monensin effects on digestibility, ruminal protein escape and microbial protein synthesis on high-fiber diets. J. Anim. Sci. 61:654–660.

Grainger, C., and K. A. Beauchemin. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Technol. 166:308–320.

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

Guyader, J., M. Eugène, B. Meunier, M. Doreau, D. P. Morgavi, M. Silberberg, Y. Rochette, C. Gerard, C. Loncke, and C. Martin. 2015. Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. J. Anim. Sci. 93:3564–3577.

Guyader, J., E. M. Ungerfeld, and K. A. Beauchemin. 2017. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). Front. Microbiol. 8:393. doi:10.3389/fmicb.2017.00393

Hino, T. 1981. Action of monensin on rumen protozoa. Jap. J. Zootech. Sci. 52: 171 – 179.

Hristov, A. N, C. Lee, R. Hristova, P. Huhtanen, and J. L Firkins. 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. J. Dairy Sci. 95:5299–5307.

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Jenkins, T. C., V. Fellner, and R. K. McGuffey. 2003. Monensin by fat interactions on trans fatty acids in cultures of mixed ruminal microorganisms grown in continuous fermenters fed corn or barley. J. Dairy Sci. 86:324–330.

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

Johnson, K. A., H. H. Westberg, B. K. Lamb, and R. L. Kincaid. 1997. Quantifying methane emissions from ruminant livestock and examination of methane reduction strategies. The Rumin. Livest. Efficiency Program Annu. Conf. Proc., EPA/USDA, Washington, DC.

Karkalas, J. 1985. An improved enzymatic method for the determination of native and modified starch. J Sci. Food Agric. 36:1019-1027.

Kluber, H. D., and R. Conrad. 1998. Inhibitory effects of nitrate, nitrite, NO and N_2O on methanogenesis by methanosarcina barkeri and methanobacterium bryantii. FEMS Microbiol. Ecol. 25:331-339.

Kone, S. O., and M. L. Galyean. 1990. Effects of combinations of monensin and lasalocid on DM disappearance, gas production and VFA productions in an in vitro ruminal culture system. Proc. West. Sect., Am. Soc. Anim. Sci. 41:402–406.

Lee, C., and K. A. Beauchemin. 2014. A review of feeding supplementary nitrate to ruminant animals: Nitrate toxicity, methane emissions, and production performance. Can. J. Anim. Sci. 94:557–570.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015a. Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers. J. Anim. Sci. 93:2391–2404.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015b. Effects of feed consumption rate of beef cattle offered a diet supplemented with nitrate ad libitum or restrictively on potential toxicity of nitrate. J. Anim. Sci. 93:4956–4965.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017. In situ and in vitro evaluation of a slow release form of nitrate for ruminants: Nitrate release rates, rumen nitrate metabolism and production of methane, hydrogen, and nitrous oxide. Anim. Feed Sci. Technol. 231:97–106.

Leng, R. A. 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A report to the department of climate change. Commonwealth Government of Australia, Canberra. www.penambulbooks.com/Downloads/Leng-Final%20 Modified%20%2017-9-2008.pdf (Accessed 14 May 2017).

Lin, M., D. M. Schaefer, W. S. Guo, L. P. Ren, and Q. X. Meng. 2011. Comparisons of in vitro nitrate reduction, methanogenesis, and fermentation acid profile among rumen bacterial, protozoal and fungal fractions. Asian-Australas J. Anim. Sci. 24:471–478.

Lund, P., R. Dahl, H. J. Yang, A. L. F. Hellwing, B. B. Cao, and M. R. Weisbjerg. 2014. The acute effect of addition of nitrate on *in vitro* and *in vivo* methane emission in dairy cows. Anim. Prod. Sci. 54:1432–1435.

Martínez-Fernández, G., L. Abecia, A. I. Martín-García, E. R. Morales, S. E. Denman, C. J. Newbold, E. M. Alcaide, D. R. Yáñez-Ruiz, and A. Stams. 2015. Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters. FEMS Microbiol. Ecol. 91:1654–1664.

Mansfield, H. R., M. I. Endres, and M. D. Stern. 1995. Comparison of microbial fermentation in the rumen of dairy cows and dual flow continuous culture. Anim. Feed Sci. Technol. 55:47–66.

Marais, J. P., J. J. Therion, R. I. Mackie, A. Kistner, and C. Dennison. 1988. Effect of nitrate and its reduction products on the growth and activity of the rumen microbial population. Br. J. Nutr. 59:301–313.

Martin, S. A., and J. M. Macy. 1985. Effects of monensin, pyromellitic diimide and 2-bromoethanesulfonic acid on rumen fermentation in vitro. J. Anim. Sci. 60: 544–550.

McAllister, T. A., S. M. S. Moustafa, K. J. Cheng, C. J. Newbold, N. McKain, and R. J. Wallace. 1994. Effect of salinomycin on fermentation and nitrogen metabolism in the artificial rumen. Can. J. Anim. Sci. 74:575–578.

McDougall, E.I., 1948. Studies on ruminant saliva. The composition and output of sheep's saliva. Biochem. J. 43:99–109.

Mir, Z. 1989. Monensin, chlortetracycline and tylosin effects on performance and digestion in lambs fed a ground alfalfa diet. Can. J. Anim. Sci. 69:505–508.

Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal. 4:1024–1036.

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

Newbold, J. R., S. M. van Zijderveld, R. B. A. Hulshof, W. B. Fokkink, R. A. Leng, P. Terencio, W. J. Powers, P. S. J. van Adrichem, N. D. Paton, and H. B. Perdok. 2014. The effect of incremental levels of dietary nitrate on methane emissions in holstein steers and performance in nelore bulls. J. Anim. Sci. 92:5032-5040.

Nocek, J. E., and J. B. Russell. 1988. Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. J. Dairy Sci. 71:2070-2107.

Nolan, J. V., I. R. Godwin, V. de Raphélis-Soissan, and R. S. Hegarty. 2016. Managing the rumen to limit the incidence and severity of nitrite poisoning in nitrate-supplemented ruminants. Anim. Prod. Sci. 56:1317–1329.

Oba, M., and M. S. Allen. 2003. Extent of hypophagia caused by propionate infusion is related to plasma glucose concentration in lactating dairy cows. J. Nutr. 133:1105–1112.

Odongo, N. E., R. Bagg, G. Vessie, P. Dick, M. M. Or-Rashid, S. E. Hook, J. T. Gray, E. Kebreab, J. France, and B. W. McBride. 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. J. Dairy Sci. 90:1781–1788.

Olijhoek, D. W., A. L. F. Hellwing, M. Brask, M. R. Weisbjerg, O. Højberg, M. K. Larsen, Jan Dijkstra, E. J. Erlandsen, and P. Lund. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. J. Dairy Sci. 99:6191-205.

Ørskov, E. R., and M. Ryle. 1990. Energy Nutrition in Ruminants. 1st ed. Elsevier Science Publishers, London.

Patra, A. K., and Z. Yu. 2014. Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures in vitro while not adversely affecting feed digestion, fermentation or microbial communities. Bioresour. Technol. 155:129–135.

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

Ricke, S. C., L. L. Berger, P. J. Van der Aar, and G. C. Fahey. 1984. Effects of lasalocid and monensin on nutrient digestion, metabolism and rumen characteristics of sheep. J. Anim. Sci. 58:194–202.

Romero-Pérez, A., E. K. Okine, L. L. Guan, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2016. Effects of 3-nitrooxypropanol and monensin on methane production using a forage-based diet in rusitec fermenters. Anim. Feed Sci. Technol. 220:67–72.

Russell, J. B., and S. A. Martin. 1984. Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms in vitro. J. Anim. Sci. 59:1329–1338.

Russell J. B., and H. J. Strobel. 1988. Effects of additives on in vitro ruminal fermentation: A comparison of monensin and bacitracin, another gram-positive antibiotic. J. Anim. Sci. 66:552–558.

Stewart, C. S., C. A. McPherson, and E. Cansunar. 1987. The effect of lasalocid on glucose uptake, hydrogen production and the solubilization of straw by the anaerobic rumen fungus neocallimastix frontalis. Lett. Appl. Microbiol. 5: 5-7.

Stewart, C. S., and A. J. Richardson. 1989. Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. J. Appl. Bacteriol. 66:85-93.

Sylvester, J. T., S. K. R. Karnati, B. A. Dehority, M. Morrison, G. L. Smith, N. R. St-Pierre, and J. L. Firkins. 2009. Rumen protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci. 92:256–269.

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

Ullrich, W. R. 1992. Transport of nitrate and ammonium through plant membranes. In: K. Mengel, D.J. Pilbeam, editors, Nitrogen Metabolism of Plants. Clarendon Press, Oxford, p. 121–137.

Ungerfeld, E. M., and R. A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: K. Sejrsen, T. Hvelplund, and M. O. Nielsen, editors, Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. Wageningen Academic Publishers, Wageningen, the Netherlands. p. 55–85.

Van Nevel, C. J., and D. I. Demeyer. 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. 34:251–257.

Van Soest, P. J., J. B. Robertson, and B.A. Lewis. 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597.

Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93:5856–5866.

van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94:4028-38.

Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, H. B. Perdok, and C. J. Newbold. 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PloS One 10: e0140282. doi: 10.1371/journal.pone.0140282

Wallace, R. J., J. W. Czerkawski, and G. Breckenridge. 1981. Effect of monensin on the fermentation of basal rations in the rumen simulation technique (RUSITEC). Br. J. Nutr. 46:131–148.

Wang, R., and N.M. Crawford. 1996. Genetic identification of a gene involved in constitutive, high-affinity nitrate transport in higher plants. Proc Natl Acad Sci USA. 93:9297–9301.

Wedegaertner, T. C., and D. E. Johnson. 1983. Monensin effect on digestibility, methanogenesis and heat increment of cracked corn-silage diet fed to steers. J. Anim. Sci. 57: 168–177

Wolin, M. J. 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43:1452–1459.

Yang, C., J. A. Rooke, I. Cabeza, and R. J. Wallace. 2016. Nitrate and inhibition of ruminal methanogenesis: Microbial ecology, obstacles, and opportunities for lowering methane emissions from ruminant livestock. Front. Microbiol. 7: 132. doi: 10.3389/fmicb.2016.00132

CHAPTER 4

NITRATE AND MONENSIN EFFECT ON ENTERIC METHANE EMISSIONS FROM GROWING STEER

Nitrate and monensin effect on enteric methane emissions from growing steers

M. Capelari,* B. Latack,* J. Roth,* and W. Powers,†[2]

§ Michigan State University, East Lansing, MI, 48824
† University of California, Oakland, CA, 94607.

[1]The authors thank CAPES foundation, Ministry of Education of Brazil, for providing funds through the scholarship number 13634/2013-5, and Yara North America Inc and Elanco Animal Health for additives donation.

[2] Corresponding author: wendy.powers@ucop.edu

4.1. Abstract

A study was conducted to evaluate the effect of calcium ammonium nitrate (**NIT**), sodium monensin (**MON**) and the combination of NIT+MON on enteric methane (**CH_4**) emissions and rumen fermentation of beef steers. Angus crossed steers (335 ± 18 kg; n=8) fed each of 4 treatment diets in a 4 × 4 replicated Latin-square (control, 1.5% NIT (DM basis), 33 mg of MON per kg of DM and the combination of NIT+MON). Four 21-d periods included a 10-d adaptation period whereby NIT was increased, stepwise, in the diet to allow acclimation of rumen microorganism to NIT supplementation. After adaptation, animals were randomly allocated to environmental rooms (1 animal per room), for 7 d for gas measurements, rumen fluid and blood sampling, with ad libitum access to water and feed. A basal diet was offered to all animals throughout the feeding period (50% high moisture corn, 30% corn silage, 15% dry distillers grains, 3% vitamin and mineral premix). Additives were mixed with a premix carrier, daily, then added to the final ration. Dry matter intake of animals fed control diet were 8.96 kg/d and tended to be reduced by MON (8.76 kg/d; $P = 0.07$) and by NIT+MON (8.35 kg/d; $P = 0.07$). Methane production from control animals was 184.4 g/d and was reduced by NIT (167.7 g/d; $P < 0.05$) and tended to be reduced by MON (176.8 g/d; $P = 0.08$). The combination of NIT+MON tended to further reduce CH_4 production when compared to animals fed either additive alone (157 g/d; $P < 0.08$). Methane yield was 20.8 g/kg DMI in control fed steers and was reduced by NIT feeding (19.1 g/kg DMI; $P < 0.05$). Total rumen VFA production from animals fed control diet was 107.9 mM and was significantly increased by NIT (114.9 mM; $P < 0.05$). Ruminal acetate concentration was greater in control fed animals (54.4 mole/100 mole) compared to MON fed animals (52.2 mole/100 mole; $P < 0.05$). Ruminal propionate concentration in control fed animals (25.9 mole/100 mole) was increased by MON (30.2 mole/100 mole; $P < 0.05$). Monensin significantly reduced acetate:propionate ratio ($P < 0.02$). Rumen NH_3 levels, when

compared to control, were reduced by MON (12.8 vs. 17.3 mg/dL; $P < 0.05$) and tended to be increased by NIT (20.1 vs. 17.3 mg/dL; $P = 0.07$). The combination of additives reduced propionate concentration when compared to MON, but other major rumen fermentation parameters were not affected. The combination of NIT+MON demonstrated potential to serve as CH_4 mitigation option for ruminants in the short term.

Key words: beef, fermentation, methane, monensin, nitrate**,** rumen

4.2. Introduction

Human activities drive emissions of greenhouse gases (**GHG**), contributing to observed warming since the mid-20th century (IPCC, 2014; Wuebbles et al., 2017). In the United States, enteric fermentation from ruminants is the largest source of anthropogenic methane (**CH_4**) emissions (25% of the total CH_4 emissions; EPA, 2014). Globally, livestock contribute 6.3% of total anthropogenic GHG emissions, with CH_4 emissions from enteric fermentation accounting for 2.1 Gt CO_2 Eq/yr and manure management accounting for 0.99 Gt CO_2 Eq/yr (IPCC, 2014). In addition, CH_4 represents a loss of 2 to 12% of diet GE (Johnson and Johnson, 1995) that could be rechanneled towards usable products for the host (Grainger and Beauchemin, 2011). Therefore, reducing CH_4 production by ruminants contributes to global efforts to reduce GHG emissions and improves the efficiency of feed use.

Rumen CH_4 formation is a disposal mechanism by which excess hydrogen (**H_2**) from anaerobic fermentation can be released (Morgavi et al., 2010). Therefore, metabolic pathways involved in H_2 production and utilization are important factors that should be considered when developing CH_4 mitigation strategies (Martin et al., 2010). When testing 2 different additives that affect the H_2 pool in the rumen, an additive effect of nitrate (**NIT**) and monensin (**MON**) on reduction of CH_4 production was reported in batch culture (Capelari and Powers, 2017) and in

semi-continuous culture trials (Capelari et al., 2017). Nitrate reduction to ammonia (**NH_3**) competes with methanogens for H_2 (Leng, 2008), and intermediates of this pathway inhibit methanogens growth (Asanuma et al., 2014). Monensin controls H_2-generating microbes and favors propionogenesis, a H_2 consuming pathway (Nagaraja et al., 1997). Hence, NIT and MON affect the H_2 pool in the rumen through different mechanisms. However, depending on dose of NIT and MON, reduction on digestion of some feed components may occur, which can be detrimental to animal performance (Capelari et al., 2017). In addition, because animals not adapted to NIT can develop methemoglobinemia (Lee and Beauchemin, 2014) it is important to evaluate potential interactions between NIT and MON in animals. We hypothesize that NIT and MON additively reduce CH_4 production. The objective of this study was to determine the effect of NIT and MON on enteric CH_4 emissions and rumen parameters of steers.

4.3. Materials and Methods

The study was conducted at Michigan State University Animal Air Quality Research Facility (**AAQRF**) and Beef Cattle Teaching and Research Facility (**BCTRC**). All procedures described below were approved and followed internal guidelines recommended by the Animal Care and Use Committee of Michigan State University (IACUC number 12/16-204-00).

4.3.1. Animals, diet and experimental design

Eight angus crossed steers (initial BW=335 Kg ± 22) were used in a replicated 4×4 Latin square design with four 21-d periods. Animals were weighed before the experiment and then grouped by BW (i.e., 4 steers per group). Forty-five d before the experiment start, all steers were adapted to human handling with 3 h of daily training. Each 21-d period consisted of 10 d (D1 to D10) for adaptation to experimental diets followed by 7 d (D11 to D17) of data collection and 5 d (D18 to D22) for wash-out period, where all animals were fed the same basal diet. The basal

diet was prepared in the form of total mixed ration (**TMR**) and consisted of approximately 50% high moisture corn, 30% corn silage, 15% corn dry distiller's grains and 5% mineral and vitamin premix. The feedstuffs were mixed using a mixer wagon (Data Ranger; American Calan, Northwood, NH) every morning. Steers in each group were allocated, randomly, to one of 4 treatments (DM basis): 1) control (**CON**), which was supplemented with 1% urea, 2) NIT, which was supplemented with 1.5% NIT in the form of calcium ammonium nitrate decahydrate (Yara International ASA, Oslo, Norway), 3) MON, which was supplemented with sodium monensin (Elanco Animal Health, Greenfield, IN) at 33 mg/kg of DM, and 4) NIT+MON, which was supplemented with both additives at 1.5% NIT and 33 mg of MON per kg of DM. The dose of NIT and MON was based on previous research (Newbold et al., 2014; Lee et al., 2015a,b; Capelari and Powers 2017; Capelari et al., 2017). Diets were formulated to meet the requirements of growing beef cattle at 1.05 kg/d ADG (National Academies of Science, Engineering, and Medicine, 2016; Table 1). Because the NIT source contained approximately 19% Ca on DM basis, limestone was added to maintain similar Ca levels across diets.

The 10-d adaptation period was conducted at BCTRC. Steers receiving NIT in the diet were adapted in a stepwise manner to avoid potential nitrate toxicity. Nitrate inclusion was increased by 10% daily until D6. From D7 onwards 100% of the dose was added. Therefore, animals fed NIT and NIT+MON treatments received the full NIT dose 3 d before entering the chambers for gas measurements. Monensin was included in the diet on D1. Steers were grouped in pairs (2 steers per pen) and diets were fed once daily at 0730 h with free access to water.

4.3.2. Sampling and measurement procedures

Throughout the experimental period, feed ingredients were sampled every 5 d. Whenever DM content changed by 3 percentage units the diets were reformulated to accommodate changes.

Experimental diets were sampled, daily, and then pooled by period for analysis of DM, OM, CP, NDF, ADF, starch, Ca, P, S. Steers BW was measured at the beginning and end of each period without fasting. Blood samples for methemoglobin (**MetHb**) analysis were sampled at D 1, 4 and 7 during the adaptation phase and on D 12 and 15 when animals were in the environmental rooms. Blood was collected 3 h after feeding in heparinized evacuated blood collection tubes and placed on ice directly after sampling. Blood was analyzed within 1 h of collection for MetHb. It was pre-established that if concentration of MetHb exceeded 20% of total hemoglobin (**Hb**), the animal would be withdrawn from the experiment as a precautionary measure. While in the environmental room, animals were observed for clinical signs of NIT poisoning during morning and afternoon health checks by analysis of oral and ocular mucosa pigmentation. Subclinical signs of methemoglobinemia usually only occur at MetHb values of 30 to 40% and higher (Bruning-Fann and Kaneene, 1993).

Rumen samples (approximately 100 mL) were collected from each steer 0, 3, and 12 h after feeding by oral lavage according to procedure described by Lodge-Ivey et al. (2009). Rumen fluid was mixed thoroughly and squeezed through 2 layers of PeCAP polyester screen (pore size 355 μm; B & SH Thompson, Ville Mont-Royal, QC, Canada). An aliquot of 25 mL of fluid was acidified with 1 mL of H_2SO_4 and frozen (−20º C) for future NH_3 analysis. A second aliquot of 10 mL was collected for offsite VFA, nitrate and nitrite analysis. Rumen fluid pH was determined on site with a pH meter (Model HQ40d Portable pH meter, BACH Co, Reno, NV). Data obtained on 0, 3 and 12 h post feeding was averaged for statistical analysis.

Measurement of gas production from steers was conducted in 12 environmentally controlled rooms (2.1 m height by 4.0 m width by 8.5 m length) designed to continuously monitor incoming and exhaust concentration of gases (Li et al., 2011). Airflow into each room

was monitored with a pressure transducer (Setra model 239; Setra Systems Inc., Boxborough, MA). Temperature and humidity were monitored using a probe (CS500; Campbell Scientific Inc., Logan, UT). Rooms were maintained under positive air pressure with average flow rate of 284 L/s. Room ventilation rate was based on animal care guidelines and the heating/cooling system that used flow rate to maintain room temperature set points. Through software control (LabVIEW Ver. 7.0, National Instruments; Austin, TX), CH_4 and N_2O concentrations were monitored in the chambers in a sequential manner, beginning first with incoming air, then sampling exhaust air from each of the 12 chambers. The incoming air line was purged for 14.5 min and chamber sample lines were purged for 9.5 min before the start of data collection. Following purging, data were collected for 5.5 min. Gas concentrations were measured simultaneously within a sample stream. Samples from the chambers were pulled to a sampling manifold using a Cole-Parmer vacuum pump (Cole-Parmer Instrument Company, Vernon Hills, Ill.) at a rate of 30 L/min, through Teflon tubing (30.43 m long with an outer diameter of 9.5 mm) placed 127 mm into the exhaust duct of each individual chamber. From the manifold the air stream was diverted into gas analyzers: a Mode 55i back-flush gas chromatography (**GC**) CH_4 analyzer (range = 0 to 100 ppm; detection limit = 0.05 mg/kg; Thermo Fisher, Franklin, MA), and a 46i photoacoustic N_2O analyzer (range = 0 to 50; ppm; detection limit = 0.02 ppm).

Methane and N_2O concentrations were calculated by mass balance of concentrations in the incoming and outflowing air using the equation (Li et al., 2011),

$$ER = Q \times (273/T) \times (C0 - C1) \times 10^{-6} \times MW/Vm,$$

in which ER is emission rate (g/min), Q is flow rate at room temperature and pressure (L/min), T is air temperature of exhaust air (°K), C0 is gas concentration in exhausting air (mg/kg), C1 is gas concentration in incoming air (mg/kg), MW is molecular weight of gas (g/mol), and Vm is

molar volume of gas at standard condition (22.414 L/mol). The emission rate (mean 1-min emissions rate for the 15-min sample cycle) was multiplied by 195 min to obtain emissions in a full cycle. For each period, the 8 steers were randomly assigned to 1 of 12 rooms thereby avoiding a confounding effect of room.

When in the environmental rooms, animals were fed once daily at 0530 h and had free access to water. Urine and feces were collected in a pan located behind the animals and manure was cleaned daily to avoid accumulation and consequent CH_4 production arising from manure.

4.3.3. Laboratory Analysis

Feed analysis from ingredients used in TMR were conducted by wet chemistry (Dairy One Forage Analysis Laboratory, Ithaca, NY). For VFA measurements, rumen fluid (10 mL) was transferred to 15-mL plastic centrifuge tubes. The tubes were initially centrifuged at 15,000 × g for 15 min at 4 °C. Five mL of the supernatant was transferred to a second set of 15-mL tubes and 1 mL of 25% m-phosphoric acid was added. After 15 min, samples were recentrifuged under the same conditions and 1.5 mL of supernatant was collected and transferred to GC vials for VFA analysis. Sample VFA profile was obtained by GC equipped with FID (GC-2010; Shimadzu, Addison, IL). Ultra-high purity He, carrier gas flow rate was set at 62 mL /min and purge flow was 3 mL /min. Oven temperature ramp was set to 150 °C for 2 min with further increments of 15 °C/min until reach 220 °C was reached.

Rumen samples for NH_3 analysis were thawed and NH_3 concentration determined using a Kjeldahl distillation apparatus (KJELTEC system 1002 distilling unit, FOSS, Eden Prairie, MN). Rumen samples for nitrate and nitrite analysis (2 mL) were filtered through a nylon syringe filter (0.22 μM pore size; VWR International Co, Radnor, PA) and concentration was measured with a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI). Blood samples for MetHb

were analyzed using a blood gas analyzer (ABL 800 Flex series; Radiometer, Copenhagen, Denmark).

4.3.4. Statistical Analysis

All data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Data of DMI, CH_4 and N_2O yield were analyzed with day as a repeated measure and the autoregressive (AR(1)) covariance structure. The following model was used:

$$Y_{i(k)jm} = \mu + k_i + \beta(k)_{ij} + T_n + P_m + e_{i(j)nm}$$

where $Y_{i(k)jm}$ is the dependent variable, μ is the overall mean, k is the random effect of group, β is the random effect of steer within group, T is the fixed effect of treatment, P is the fixed effect of period and e is the error term assumed to be normally distributed with mean = 0 and constant variance. Statistical differences were declared at $P \leq 0.05$. Differences between treatments with $0.05 < P < 0.10$ were considered as a trend toward significance. Data are presented as least squares means.

4.4. Results

No animals were removed from the study due to blood MetHb concentration. Animals gained, on average, 26.4 kg of BW per experimental period, with a final average BW of 444.5 kg. There was no difference in blood MetHb concentration among treatments (Table 2). The highest MetHb concentration observed was 4% for a single animal fed the NIT treatment (data not shown).

Dry matter intake averaged 8.72 kg DM/d and tended to be reduced in steers fed MON (8.76 kg/d) and NIT+MON (8.35 kg/d; $P = 0.07$; Table 3). Compared with the CON diet, CH_4

(g/d) was 9, 5, and 15% less from steers fed the NIT, MON, and NIT+MON diets, respectively. There was a trend for an additive CH_4–mitigating effect between NIT and MON (nitrate × monensin, $P = 0.08$). Methane yield for CON was 20.8 g/kg DMI. Nitrate significantly reduced CH_4 yield (19 g/Kg DMI; $P = 0.05$), but MON did not affect CH_4 yield (20.1 g/kg DMI).

Treatments did not affect rumen pH, which average 6.4 ($P > 0.05$; Table 4). Total VFA production was significantly increased by NIT (114.9 mM) and NIT+MON (113.5 mM) when compared to control (107.9 mM; $P < 0.05$). Monensin, when compared to control, significantly increased propionate (30.2 vs. 25.9 mol/100mol; $P < 0.05$) while reducing acetate (52.5 vs. 54.4 mol/100 mol; $P < 0.05$) and acetate:propionate ratio (1.8 vs. 2.3; $P < 0.05$). There was a trend for NIT, when compared to control, to reduce propionate concentration (23.8 vs. 25.9 mol/100mol; $P = 0.07$). No other significant effects of treatments on other major VFA was observed. Rumen NH_3 levels, when compared to control, were reduced by MON (12.8 vs. 17.3 mg/dL; $P < 0.05$) and tended to be increased by NIT (20.1 vs. 17.3 mg/dL; $P = 0.07$).

4.5. Discussion

4.5.1. Nitrate Toxicity

In the rumen NIT reduction occurs by dissimilatory and assimilatory processes. Dissimilatory NIT reduction occurs primarily via a two-step pathway where NIT is first reduced to NO_2, which can accumulate as an intermediate before it is ultimately reduced to NH_3 (Latham et al., 2016). If NO_2 accumulates in the rumen, it can be absorbed into the blood through the rumen wall. As a result of NO_2 in the blood, methemoglobin is incapable of oxygen transport, that converts ferrous iron into ferric iron (Ozmen et al., 2005). When high amounts of NIT are fed to ruminants, the capacity of the rumen flora to completely reduce NIT may be overwhelmed, resulting in a pool of NO_2 in the rumen and absorption of NO_2 from the rumen

into blood. Factors related to NIT poisoning include: 1) NIT level in the diet, 2) NIT consumption rate, 3) incomplete NIT and NO_2 reduction to NH_3 in the rumen and 4) slow rumen passage rate (e.g., longer nitrate or nitrite retention in the rumen; Leng, 2008; Lee and Beauchemin, 2014).

No sign of NIT poisoning were observed byeither by MetHb concentration in blood, through evaluation of clinical symptoms during daily health checks (e.g., oral and ocular mucosa pigmentation), or by performance variables such as DMI and BWG. The level of NIT in this experiment (1.5% of diet DM) is lower than recent studies where animals were withdrawn from experiment due to high MetHb levels (3% of NIT in diet DM; Newbold et al., 2014). Experimental dose tested is also an intermediary level compared with those previously tested *in vitro* in batch culture when NIT increased production of nitrous oxide (N_2O), an intermediate of NIT dissimilatory reduction (Capelari and Powers, 2017), and semi-continuous culture when NIT and NO_2 were detected in rumen fluid after 24 h of feeding (Capelari et al., 2017). Highly fermentable diets, such as the one fed in this experiment, may provide protection against NIT intoxication (Burrows et al.,1987; Takahashi et al., 1980). The potential for toxicity of NIT can be reduced by acclimatizing animals to NIT gradually (Lee and Beauchemin, 2014), and studies supplementing NIT up to 3% of diet DM have been performed without signs of toxicity (van Zijderveld et al. 2010, 2011; Guyader et al., 2015; Lee et al., 2015a,b). Although the rumen ecosystem can acclimate to NIT, alternative technologies have been developed. Propionibacteria have been proposed as direct-fed microbials to reduce the risk of NIT toxicity (Swartzlander, 1994). An encapsulated form of NIT tested *in vitro* (Capelari et al., 2017; Guyader et al., 2017) and *in vivo*, showed no signs of NIT poisoning (El-Zaiat et al., 2014; Lee et al., 2015a,b) or reduction in animal performance (Lee et al., 2017a,b).

4.5.2. Methane emissions

Supplemental NIT is generally considered an effective agent that reduces enteric CH_4 emissions from ruminants (Hristov et al., 2013), and despite concerns with NIT poisoning, NIT has received attention as an electron acceptor in the rumen that could reduce enteric CH_4 emissions. Several *in vivo* studies confirmed the efficacy of feeding nitrate on reducing enteric CH_4 emissions in sheep (Nolan et al. 2010; van Zijderveld et al. 2010), dairy (van Zijderveld et al. 2011; Guyader et al., 2015) and beef cattle (Lee et al., 2015a, b) without resulting in clinical signs of toxicosis. In this experiment, NIT fed alone reduced CH_4 levels by 9 percent when compared to control. During NIT reduction to NH_3 in the rumen, 4 moles of H_2 are consumed. Stoichiometrically, 100 g of NIT should lower CH_4 emissions by 25.8 g, assuming all NIT is reduced to NH_3. In the current study, CH_4 mitigation was 12.65 g/d per percentage unit dietary NIT, representing an efficacy of 49 percent. This efficacy is low, but similar to what Newbold et al. (2014) observed when feeding Holstein steers with 5 incremental levels of NIT. Hulshof et al. (2012) observed 87 percent efficacy when beef cattle were fed a sugarcane-based diet with 2.2 percent NIT on a DM basis. Lee et al. (2015a) fed beef heifers with 1, 2 and 3% encapsulated nitrate in the diet DM and observed 35, 52 and 83 percent efficacies of CH_4 mitigation, which contrasts van Zijderveld (2011) observation that CH_4 mitigation efficacy is negatively correlated with levels of NIT consumed (5 studies and 9 theoretical efficacies; $R^2 = 0.85$). The most likely cause of inefficient CH_4 mitigation is incomplete NIT reduction to NH_3. If H_2 utilized during NIT reduction is diverged from propionogenesis rather than methanogenesis it can contribute to reduced efficacy. It is possible that in diets containing high levels of starch, which favors the production of propionate, H_2 consumed for NIT reduction is diverged from propionogenesis rather than methanogenesis. Nitrate tended to decreased propionate concentration when compared to CON in this experiment. In addition, NIT can be absorbed into the blood and be

excreted in the urine or pass the rumen and be excreted in feces. Lee et al. (2015a) observed 0.6 and 0.3% of ingested NIT was excreted in urine and feces, respectively, when NIT was fed at 3% diet DM. Lewis (1951) found that approximately 8 percent of a nitrate load introduced directly into the rumen was recovered in urine.

Monensin is a carboxylic polyether ionophore, commonly used to improve efficiency of energy (Byers, 1980) and N utilization (Ruiz et al., 2001) in cattle. Bergen and Bates (1984) categorized rumen fermentation alterations associated with ionophore feeding into 3 major areas: 1) increased production of propionate and reduction of CH_4, resulting in increased efficiency of energy metabolism of the rumen and/or animal, 2) reduced protein degradation and deamination of amino acids, resulting in the improvement of N metabolism in the rumen and/or animal, and 3) reduced lactic acid production and froth formation in the rumen, leading to reduction of ruminal disorders. Monensin, when fed alone, tended to decrease CH_4 emissions when compared to control diets by lowering CH_4 production by 5 percent. This represents a reduction in CH_4 output of 7.6 g/d and is similar to that observed by McGinn et al. (2004), who fed Holstein steers with a barley silage-based diet and 33 mg of MON per kg of DM. The reduction observed when MON was fed in the present study was in agreement with Beauchemin et al. (2008), who found evidence of a dose response effect of MON on CH_4 emission, with MON at < 19 mg/kg of diet DM not reducing CH_4 emissions, but 24 to 35 mg/kg of DM reducing CH_4 by 3 to 8 percent. In a recent meta-analysis of 22 controlled studies, Appuhamy et al. (2013) observed that CH_4 reduction effect of MON ranges between 12 ± 6 g/d in dairy cows and 14 ± 6 g/d in beef steers, with mitigation outcomes similar when adjusted for dose differences between dairy and beef studies. In addition, the effect of MON on CH_4 reduction is likely transient, as suggested by Guan et al. (2006) who observed concomitant return of CH_4 output and protozoa numbers to

background levels in the initial 4 wks of supplementation. Therefore, it is possible that greater reductions could be observed if CH_4 was measured in the initial 10 d following MON supplementation. The combination of NIT and MON reduced CH_4 production (g/d) by 15% when compared to control, and further reduce emissions when compared to either additives individually. Part of this decrease is likely related to the reduction in DMI observed when additives were combined.

4.5.3. Rumen fermentation profile

Previous *in vitro* studies reported that when NIT and MON were combined in batch culture and semi-continuous culture of rumen microorganisms the most significant change in the fermentation profile was a reduction in CH_4 production with concomitant increase in proportion of propionate in detriment of acetate (Capelari and Powers, 2017; Capelari et al., 2017). The most consistent and well documented alteration caused by MON on ruminal fermentation is the increased molar proportion of propionate with a concurrent decrease in the molar proportion of acetate and butyrate. We observed this alteration with a consequent reduction in acetate:propionate ratio with no significant change in total VFA production. However, when NIT and MON were combined, propionate concentration decreased when compared to MON alone, likely due to the incorporation of H_2 in NIT reduction products rather than propionate formation.

Total VFA production was increased by NIT with a significant increase in acetate. An increase in total VFA production was also observed by Olijhoek et al (2015) when dairy cows were supplemented with 4 levels of NIT. Troy et al. (2015) fed beef cattle with NIT and also reported an increase in acetate and a decrease in propionate in beef cattle fed two contrasting diets. Thermodynamically, reduction of NIT is more favorable than other major H_2 sinks, and the process preferentially directs H_2 away from methanogenesis, but could also draw H_2 away from

other processes such as propionogenesis (van Zijderveld et al., 2010) and fatty acid biohydrogenation (Lourenço et al., 2010). Propionate concentration may change because NIT is an alternative H_2 acceptor to endogenous fumarate in many propionate-producing bacteria (Yang et al., 2016). Thus, NO_2 is formed rather than succinate, which would then be decarboxylated to propionate, and the balance of VFA moves away from propionate. Following absorption, propionate is the only VFA that is glucogenic, so a lower molar propionate production rate would generally be considered detrimental to nutrition (Leng et al., 1967), although excess propionate can also lead to undesirable consequences such as reduction in voluntary intake in dairy cows (Oba and Allen, 2003; Allen et al., 2009) and low milk fat content and fat syndrome in lambs (Ørskov and Ryle, 1990).

4.5.4. Nitrate as a Feed Additive for Ruminants

Nitrates may be present in the diet of ruminants on a continuous basis, usually in the form of nitrate salts and ammonium nitrate. Nitrate uptake by plants is an essential step in the incorporation of soil N (Burrows et al., 1987) and the most common source of NIT in the diet of ruminants are forages from perennial ryegrass and the sorghum family (sorghum, sudan, pearl millet, and their crosses; Leng, 2008). Factors affecting NIT concentration in forage have been well demonstrated by Leng (2008) and include 1) forage maturity, 2) soil conditions (e.g., moisture content), and 3) application of fertilizers to soil. Nevertheless, supplemental NIT is not permitted in cattle feed in the United States and Canada (National Academies of Science, Engineering, and Medicine, 2016). However, in Australia carbon credits are now available for managed feeding of NIT for grazing ruminants (Department of Environment, Australian Government, 2015). Acceptance of NIT feeding by producers as a means of gaining carbon credits from reduced CH_4 output by their livestock will depend on whether they can achieve the

nutritional benefits of non-protein N supplementation using NIT at a cost similar to that of urea supplementation and with a minimal risk of NO_2 poisoning or other adverse effects on animal production (Nolan et al., 2016).

In summary, NIT alone, MON alone and NIT+MON reduced CH_4 by 9, 5 and 15 percent, respectively, demonstrating that NIT has potential to serve as a mitigation option for ruminants. The observed reduction in CH_4 was significant but lower than that previously reported in semi-continuous culture when both additives (1.25% of NIT and 4 mg/L of MON) were combined and reduced, on average, 40 percent of CH_4 production (Capelari et al., 2017). Nitrate reduced CH_4 at low efficacy when compared to other studies which suggests that effectiveness of NIT to reduce CH_4 could be influenced by other factors such as diet composition. No signs of nitrate toxicity were observed during the experiment. The increase in propionate observed when MON was fed alone was reduced for NIT+MON, likely because NIT reduction and propionogenesis compete for metabolic H_2. Studies to evaluate the performance of ruminants when fed the combination of additives are needed.

Table 4.1. Feed ingredients and chemical composition of the diets

Item	CON	NIT	MON	MON+NIT
Ingredients (DM, %)				
High moisture shell corn[1]	50.0	50.0	50.0	50.0
Corn silage[2]	30.0	30.0	30.0	30.0
Corn dry distillers grains[3]	15.0	15.0	15.0	15.0
Supplements				
Limestone	1.0	0.0	1.0	0.0
Premix (min and vit)[4]	3.0	3.0	3.0	3.0
Monensin source (mg/Kg of DM)[5]	0.0	0.0	33.0	33.0
Nitrate source[6]	0.0	2.1	0.0	2.1
Urea	0.9	0.0	0.9	0.0
Chemical composition (% of DM)				
DM, % of as fed	47.7	47.7	47.7	47.7
CP	13.3	13.2	13.3	13.3
NDF	23.2	23.2	23.2	23.2
ADF	11.9	11.9	11.9	11.9
Ca	1.3	1.3	1.	1.3
P	0.4	0.4	0.4	0.4
Ca: P	3.4	3.3	3.4	3.3
ME (Mcal/kg DM)	3.2	3.2	3.2	3.2

[1]DM = 65.3%; CP = 7.6%; NDF = 6.9%; ADF = 2.5%; NFC = 79.9%.

[2]DM = 27.2%; CP = 7.3%; NDF = 48.7%; ADF = 29.4%; NFC = 35.5%.

[3]DM = 87%; CP = 34%; NDF = 34.6%; ADF = 12.8%; NFC = 15.0%.

[4]Mg, 90 g/kg DM; S, 40 g/kg DM; Co, 0.2 g/kg DM; Cu, 10 g/kg DM; I, 0.9 g/kg DM; Fe, 20 g/kg DM; Mn, 40 g/kg DM; Se, 0.3 g/kg DM; Zn, 40 g/kg DM; vitamin A, 4,400,000 IU/kg DM; vitamin D3, 550,000 IU/kg DM; and vitamin E, 5,500 IU/kg DM.

[5]Rumensin 90 (90.5 g of active drug per 453 g of product DM; Elanco Animal Health, Greenfield, IN).

[6]The source of nitrate was the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)2 \cdot NH_4NO_3 \cdot 10H_2O$]; 85.6% DM; 17.6% N, 19.6% Ca, and 71.4% nitrate on a DM basis. Yara International ASA, Oslo, Norway.

Table 4.2. Effects of nitrate (NIT), monensin (MON) and the combination of additives on BW gain and blood hemoglobin and methemoglobin levels of steers

						P – value[3]		
Item	CON	NIT[1]	MON[2]	NIT+MON	SEM	NIT	MON	NIT × MON
BW, kg (start)	338	332	336	335	15	NS	NS	NS
BW, kg (end)	443	437	447	451	21	NS	NS	NS
Hb (g/dL)	11.8	11.6	11.6	11.7	0.7	NS	NS	NS
MetHb (% of Hb)	1.2	1.4	1.2	1.2	0.09	NS	NS	NS

[1]Calcium ammonium nitrate decahydrate (0 and 1.5 % of diet DM; Yara International ASA, Oslo, Norway).

[2]Sodium monensin (0 and 33 mg/kg of diet DM; Elanco Animal Health, Greenfield, IN).

[3]NS = non significant ($P > 0.05$).

Table 4.3. Effects of nitrate (NIT) and sodium monensin (MON) on enteric CH_4 emission and CH_4 yield

Item	CON	NIT[1]	MON[2]	NIT+MON	SEM	P – value[4]		
						NIT	MON	NIT × MON
DMI[3]	8.96^{a}	8.81^{ab}	8.76^{b}	8.35^{b}	0.28	NS	0.06	0.08
CH_4 g/d	184.4^{a}	167.7^{c}	176.8^{b}	157.0^{d}	13.1	<0.05	0.06	0.08
CH_4 g/kg DMI	20.8^{a}	19.0^{b}	20.1^{a}	18.7^{b}	1.1	0.05	NS	NS
CH_4 L/d	257.5^{a}	234.3^{c}	246.9^{b}	219.3^{d}	15.3	<0.05	0.08	0.08
CH_4 L/kg DMI	29.1^{a}	26.7^{b}	28.2^{a}	26.3^{b}	1.6	0.05	NS	NS

$^{a-e}$ means within row with different superscript differ ($P < 0.01$).

[1]Calcium ammonium nitrate decahydrate (0 and 1.8 % of diet DM; Yara International ASA, Oslo, Norway).

[2]Sodium monensin (0 and 33 mg/ Kg of diet DM; Elanco Animal Health, Greenfield, IN).

[3]DMI measured when steers were in the chambers.

[4]NS = non significant ($P > 0.05$).

Table 4.4. Effect of nitrate (NIT) and sodium monensin (MON) on rumen fermentation profile of steers

Item	CON	NIT[1]	MON[2]	NIT+MON	SEM	*P*- value[3]		
						NIT	MON	NIT × MON
pH	6.31	6.41	6.45	6.36	0.5	NS	NS	NS
Total VFA, mM	107.9[b]	114.9[a]	108.1[b]	113.5[a]	2.92	0.04	NS	NS
VFA composition, mol/100 mol								
Acetate (A)	54.4[a]	55.1[a]	52.2[b]	53.1[b]	0.6	NS	0.04	NS
Propionate (P)	25.9[b]	23.8[b]	30.2[a]	28.1[a]	1.7	0.09	0.04	NS
Butyrate	13.7	13.8	12.4	13.1	1.3	NS	NS	NS
Valerate	1.5	1.7	1.4	1.4	0.1	NS	NS	NS
Caproate	0.3	0.4	0.7	0.8	0.1	NS	0.06	NS
Heptanoate	0.6	0.8	0.6	0.6	0.1	NS	NS	NS
Isobutirate	1.1	1.4	1.4	1.3	0.5	NS	NS	NS
Isovalerate	2.1	2.5	2.6	2.2	0.1	NS	NS	0.08
A:P	2.3[a]	2.5[a]	1.8[b]	1.9[b]	0.1	NS	0.02	NS
NH_3, mg/dL	37.3[a]	40.0[a]	29.9[b]	40.8[a]	3.9	0.07	<0.05	NS

[a - e]means within row with different superscript differ ($P < 0.05$).

[1]Calcium ammonium nitrate decahydrate (0 and 1.5 % of diet DM; Yara International ASA, Oslo, Norway).

[2]Sodium monensin (0 and 33 mg/ Kg of diet DM; Elanco Animal Health, Greenfield, IN).

[3]NS = non significant ($P > 0.1$).

LITERATURE CITED

LITERATURE CITED

Allen, M. S., B. J. Bradford, and M. Oba. 2009. Board-invited review: The hepatic oxidation theory of the control of feed intake and its application to ruminants. J. Anim. Sci. 87:3317-3334.

Appuhamy, J. A. D. R. N., A. B. Strathe, S. Jayasundara, C. Wagner-Riddle, J. Dijkstra, J. France, and E. Kebreab. 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. J. Dairy Sci. 96:5161-5173.

Asanuma, N., S. Yokoyama, and T. Hino. 2014. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to Selenomonas ruminantium having the ability to reduce nitrate and nitrite. Anim. Sci. J. 86:378–384.

Beauchemin, K. A., M. Kreuzer, F. O'Mara, and T.A. McAllister, 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48:21–27.

Bergen, W. G., and D. B. Bates. 1984. Ionophores: Their effect on production efficiency and mode of action. J. Anim. Sci. 58:1465–1483.

Bruning-Fann, C. S., and J. B. Kaneene. 1993. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. Vet. Hum. Toxicol. 35:237–253.

Burrows, G. E., G. W. Horn, R. W. McNew, L. I. Croy, R. D. Keeton, and J. Kyle. 1987. The prophylactic effect of corn supplementation on experimental nitrate intoxication in cattle. J. Anim. Sci. 64:1682–1689.

Byers, F. M. 1980. Determining effects of monensin on energy value of corn silage diets for beef cattle by linear or semi-log methods. J. Anim. Sci. 51:158–169.

Capelari, M., and W. Powers. 2017. The effect of nitrate and monensin on *in vitro* ruminal fermentation. J. Anim. Sci. 95:5112–5123.

Capelari, M., K. A. Johnson, B. Latack, J. Roth, and W. Powers. 2017. The effect of two additives on ruminal fermentation using a semi-continuous culture system. J. Anim. Sci. 95 (Suppl. 4): 274 (Abstr.).

El-Zaiat, H. M., R. C. Araujo, Y. A. Soltan, A. S. Morsy, H. Louvandini, A. V. Pires, H. O. Patino, P. S. Correa, and A. L. Abdalla. 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. J. Anim. Sci. 92:2214–2224.

EPA, United States Environmental Protection Agency. 2014. Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990–2012. U.S. Environmental Protection Agency, Washington, DC.

Grainger, C., and K. A. Beauchemin. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Technol. 166:308–320.

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

Guyader, J., M. Eugène, B. Meunier, M. Doreau, D. P. Morgavi, M. Silberberg, Y. Rochette, C. Gerard, C. Loncke, and C. Martin. 2015. Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. J. Anim. Sci. 93:3564–3577.

Guyader, J., E. M. Ungerfeld, and K. A. Beauchemin. 2017. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). Front. Microbiol. 8:393. doi:10.3389/fmicb.2017.00393

Hristov, A. N., J. Oh, J. L. Firkins, J. Dijkstra, E. Kebreab, G. Waghorn, H. P. S. Makkar, A. T. Adesogan, W. Yang, C. Lee, P. J. Gerber, B. Henderson, and J. M. Tricarico. 2013. Special Topics-Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci. 91:5045–5069.

Hulshof, R. B. A., A. Berndt, W. J. J. Gerrits, J. Dijkstra, van Zijderveld S. M, J. R. Newbold, and H. B. Perdok. 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci. 90:2317-2323.

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

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

Latham, E. A., R. C. Anderson, W. E. Pinchak, and D. J. Nisbet. 2016. Insights on alterations to the rumen ecosystem by nitrate and nitrocompounds. Front. Microbiol. 7:228. doi: 10.3389/fmicb.2016.00228.

Lee, C., and K. A. Beauchemin. 2014. A review of feeding supplementary nitrate to ruminant animals: Nitrate toxicity, methane emissions, and production performance. Can. J. Anim. Sci. 94:557–570.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015a. Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers. J. Anim. Sci. 93:2391–2404.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015b. Effects of feed consumption rate of beef cattle offered a diet supplemented with nitrate ad libitum or restrictively on potential toxicity of nitrate. J. Anim. Sci. 93:4956–4965.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017a. Effects of encapsulated nitrate on growth performance, nitrate toxicity, and enteric methane emissions in beef steers: Backgrounding phase. J. Anim. Sci. 95:3700–3711.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017b. Effects of encapsulated nitrate on growth performance, carcass characteristics, nitrate residues in tissues, and enteric methane emissions in beef steers: Finishing phase. J. Anim. Sci. 95:3712–3724.

Leng, R. A., J. W. Steel, and J. R. Luick. 1967. Contribution of propionate to glucose synthesis in sheep. Biochem. J. 103:785–790.

Leng, R. A. 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A report to the department of climate change. Commonwealth Government of Australia, Canberra. www.penambulbooks.com/Downloads/Leng-Final%20 Modified%20%2017-9-2008.pdf (Accessed 14 May 2017).

Lewis, D. 1951. The metabolism of nitrate and nitrite in the sheep; the reduction of nitrate in the rumen of the sheep. Biochem. J. 48:175–180.

Lodge-Ivey, S. L., J. Browne-Silva, and M. B. Horvath. 2009. Technical note: Bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. J. Anim. Sci. 87:2333–2337.

Li, W., W. Powers, and G. M. Hill. 2011. Feeding distillers dried grains with solubles and organic trace mineral sources to swine and the resulting effect on gaseous emissions. J. Anim. Sci. 89:3286–3299.

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

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

Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal. 4:1024–1036.

Martin C., D. P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal. 4:351-365.

Nagaraja, T. G., C. J. Newbold, C. J. Van Nevel, and D. I. Demeyer. 1997. Manipulation of ruminal fermentation. In: P. N. Hobson and C.S. Stewart, editors, The Rumen Microbial Ecosystem. 2nd. Ed. Blackie Academic & Professional, London, UK. p. 523–632.

National Academies of Science, Engineering, and Medicine. 2016. Nutrient requirements of beef cattle. 8th ed. Natl. Acad. Press, Washington, DC. doi:10.17226/19014.

Newbold, J. R., S. M. van Zijderveld, R. B. A. Hulshof, W. B. Fokkink, R. A. Leng, P. Terencio, W. J. Powers, P. S. J. van Adrichem, N. D. Paton, and H. B. Perdok. 2014. The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. J. Anim. Sci. 92:5032-5040.

Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2010. Effects of dietary nitrate on fermentation, methane production, and digesta kinetics in sheep. Anim. Prod. Sci. 50:801–806.

Olijhoek, D. W., A. L. F. Hellwing, M. Brask, M. R. Weisbjerg, O. Højberg, M. K. Larsen, J. Dijkstra, E. J. Erlandsen, and P. Lund. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. J. Dairy Sci. 99:6191–6205.

Oba, M., and M. S. Allen. 2003. Extent of hypophagia caused by propionate infusion is related to plasma glucose concentration in lactating dairy cows. J. Nutr. 133:1105–1112.

Ørskov, E. R., and M. Ryle. 1990. Energy Nutrition in Ruminants. 1st ed. Elsevier Science Publishers, London.

Ozmen, O., F. Mor, S. Sahinduran, and A. Unsal. 2005. Pathological and toxicological investigations of chronic nitrate poisoning in cattle. Toxicol. Environ. Chem. 87:99–106

Ruiz, R., G. L. Albrecht, L. O. Tedeschi, G. Jarvis, J. B. Russell, and D. G. Fox. 2001. Effect of monensin on the performance and nitrogen utilization of lactating dairy cows consuming fresh forage. J. Dairy Sci. 84:1717–1727.

Swartzlander, J. H. 1994. Selection, establishment and in vivo denitrification of a Propionibacterium strain in beef cattle. M.S. Thesis, Oklahoma State Univ., Stillwater.

Takahashi, J., T. Matsuko, S. Endo, K. Dodo, and H. Fujita. 1980. Effects of dietary protein and energy levels on the reduction of nitrate and nitrite in the rumen and methemoglobin formation in sheep. Jpn. J. Zootech. Sci. 51:626–632.

Troy, S. M., C-A Duthie, J. J. Hyslop, R. Roehe, D. W. Ross, R. J. Wallace, A. Waterhouse, and J. A. Rooke. 2015. Effectiveness of nitrate addition and increased oil content as methane mitigation strategies for beef cattle fed two contrasting basal diets. J. Anim. Sci. 93: 1815–1823.

van Zijderveld, S. M. 2011. Dietary Strategies to Reduce Methane Emissions from Ruminants. PhD Thesis. Wageningen Univ., Wageningen, the Netherlands.

van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93:5856–5866.

van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94:4028–4038.

Yang, C., J. A. Rooke, I. Cabeza, and R. J. Wallace. 2016. Nitrate and inhibition of ruminal methanogenesis: Microbial ecology, obstacles, and opportunities for lowering methane emissions from ruminant livestock. Front. Microb. 7: 132. 10.3389/fmicb.2016.00132.

Wuebbles, D. J., D. W. Fahey, K. A. Hibbard, B. DeAngelo, S. Doherty, K. Hayhoe, R. Horton, J. P. Kossin, P. C. Taylor, A. M. Waple, and C. P. Weaver. 2017. Executive summary. In: Wuebbles, D. J., D. W. Fahey, K. A. Hibbard, D. J. Dokken, B. C. Stewart, and T. K. Maycock, editors, Climate Science Special Report: Fourth National Climate Assessment, Volume I. U.S. Global Change Research Program, Washington, DC. p. 12-34.

CHAPTER 5

SUMMARY

The objective of this study was to quantify the benefits of supplementary nitrate and monensin in terms of CH_4 abatement in ruminants. The hypothesis was that the combination of additives would additively reduce CH_4 production without affecting health and performance parameters. This hypothesis was based on available data from rumen fermentation studies and on thermodynamic concepts applied to ruminant nutrition and production of VFA and other fermentation products in the rumen. Specific objectives included the determination of dose-related response of additives to achieve:

- Significant reduction in total CH_4 production;
- Uncompromised animal health; and
- Unchanged animal performance parameters.

In Chapter 2 we monitored ruminal fermentation and by-products profile (VFA, CO_2, CH_4, N_2O, NH_3) with a short-term *in vitro* batch culture model in order to test our hypothesis and to better understand dose-response effect of additives. The combination of additives in a closed system reduced CH_4 production by approximately 90 percent in two contrasting diets (100 percent forage diet and 10:90 forage to concentrate diet). Digestion of diet DM was reduced when additives were combined at higher doses. In addition, this trial was one of the first scientific investigations to observe increased N_2O following nitrate supplementation. Independent of diet composition, N_2O concentration significantly increased when nitrate was present and a combination of nitrate and monensin further increased N_2O production.

A second specific objective was to determine if dietary nitrate inclusion could sustain CH_4 levels below control if microbial adaptation to monensin occur. A long-term semi-continuous culture of mixed rumen microorganisms was used (Chapter 3) to further detail the effect of additives on the variables of interest in two contrasting diets (50:50 and 15:85 forage to

concentrate diets) commonly fed in dairy and beef operations across the USA. Seven 37-d trials were conducted and the results supported our hypothesis that CH_4 would be additively reduced by the combination of nitrate and monensin. We did not observe signs of microbial adaptation to monensin based on the fermentation variables measured. The reduction on CH_4 production observed when monensin was fed was maintained throughout the 30-d experimental period along with increased propionate production. Based on results obtained in Chapter 2 and 3, the effect of additives on CH_4 production was maintained independently of diet composition, demonstrating that this strategy is likely to be effective in reducing CH_4 irrespective of diet composition.

Supported by data obtained in Chapter 2 and 3, we conducted an *in vivo* trial with growing beef steers to confirm the benefits of additives on CH_4 abatement. Significant CH_4 emissions reduction was observed as a result of nitrate inclusion. Because nitrate was fed at 1.5% of diet DM, a conservative concentration compared to other studies, nitrate alone reduced CH_4 by 10 percent. Nitrate reduced CH_4 at 12.65 g/d per percentage unit dietary nitrate, representing an efficacy of 49 percent. This efficacy is low if compared to other studies. Lower efficacy is often attributed to incomplete nitrate reduction in the rumen, but blood methemoglobin levels were not different among treatments. A concentrate diet favors faster passage of nitrate that may not have been metabolized by rumen microbes. Monensin reduced CH_4 production by approximately 5 percent and increased propionate production, which is in accordance with other studies with similar diet and animal productivity. When both additives were included, CH_4 was reduced 15 percent,which was partially associated with reduced intake. *In vitro* models can serve as tools for rumen fermentation studies, but do not account for animal factors that can influence responses as observed by the different range in some variables when comparing treatment effects using *in vitro* vs. *in vivo* models. The experiments conducted in Chapters 2, 3 and 4 demonstrated the

combined nitrate and monensin mitigation potential with significant changes in fermentation parameters. More animal studies are needed to confirm the effect on the long-term and in different feeding conditions.