Evaluation of 3-nitrooxypropanol to decrease enteric methane emissions in beef cattle

by

Atmir Romero

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Animal Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

The main objective of this research was to evaluate the potential of 3-nitrooxypropanol (NOP) to lower enteric methane (CH_4) production by ruminants. Methane is an undesirable byproduct of enteric fermentation that represents a loss of energy to the animal. Additionally, CH_4 is a greenhouse gas that contributes to global warming. 3-Nitrooxypropanol is a novel compound that inhibits methyl-CoM reductase, a key enzyme of methanogenesis. Four experiments were conducted using either beef cattle or the rumen simulation technique (Rusitec). The first study evaluated the addition of increasing levels of NOP [0, 0.75, 2.25 and 4.50 mg/kg of body weight (BW)] to a beef cattle diet. Enteric CH_4 production was linearly decreased with increasing NOP dose with 33% less CH_4 at the highest level of supplementation. There was a shift in rumen fermentation towards more propionate and less acetate concentration in the rumen with NOP addition. However, NOP did not affect BW gain, feed digestibility or the numbers of of rumen bacteria, protozoa or methanogens, but slightly decreased dry matter intake (DMI). In the second experiment, the long-term (112 d) addition of NOP (2 g/d) to a beef cattle diet resulted in 60% less enteric CH_4 production compared to the control with no signs of microbial adaptation. Total numbers of methanogens and the proportion of acetate in the rumen were lowered, while the proportion of propionate was increased. This study included a recovery period (16 d) in which NOP addition was discontinued. During this period the residual effects of NOP on the variables studied were either nonexistent or minimal. The third study evaluated different NOP doses (0, 5, 10 and 20 mg/d) using Rusitec fermenters. Methane was linearly and quadratically decreased on average by 82% compared with NOP addition with no effect on feed digestibility; however, CH_4 reduction was accompanied with hydrogen gas accumulation. In this study, total methanogens associated with the solid phase (feed residuals) were decreased with

NOP addition; but, methanogens associated with the liquid phase were not affected. The fourth study evaluated the effects of NOP (2 mg/d), monensin (MON; 2 mg/d) and the combination of NOP (2 mg) and MON (2 mg) using the Rusitec system. Addition of NOP decreased CH_4 production by 71.5% and MON by 11.8% when compared to the control treatment with no additive reduction in CH_4 when the two compounds were combined. This study included a recovery period at the end of the experiment in which treatments were discontinued. During this period a gradual increse in CH_4 production was observed for NOP or NOP plus MON treatments, which approached control levels 3 d after treatment withdrawal. In conclusion, NOP is an effective means of mitigating enteric CH_4 emissions from beef cattle during prolonged feeding periods with no evidence of microbial adaptation. The reduction of CH_4 production observed in the *in vivo* experiments together with increased propionate proportion, a small reduction in DMI and no effect on BW are encouraging and open the possibility to further evaluate NOP with larger number of animals under farm conditions.

DEDICATION

To Diana, my lovely wife, and Constanza, our beloved daughter, for your patience and support through these years.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Drs. Karen Beauchemin and Erasmus Okine. Dr. Beauchemin gave me the great opportunity of working in her lab, a place with an excellent atmosphere to do research. For me, Dr. Beauchemin is the best example of how a research scientist should be. Dr. Okine gave me the opportunity to study at the University of Alberta and provided me strong support since the first day I arrive in Canada. The, guidance, help and encouragement Drs. Beauchemin and Okine provided me through my doctoral program was essential to conclude this thesis work and I am very thankful for it. I could not have imagined having better supervisors.

Sincere appreciation is extended to the rest of my supervisory committee, Drs. Tim McAllister, Sean McGinn and Masahito Oba for their insightful advice, for their encouragement and for reviewing manuscripts.

Special thanks are sent to Dr. Carolyn Fitzsimmons for agreeing to be my examainer and to Dr. Emilio Ungerfeld for serving as my external examiner.

My sincere thanks also go to Drs. Leluo Guan, Stephane Duval and Maik Kinderman who co-authored the published manuscripts in my thesis.

I would also like to thank technicians, postdoctoral fellows, students and the barn crew at the Lethbridge Research Centre and at the University of Alberta. Without their help it would not be possible to have realized this project.

In addition, I would also like to thank Agriculture and Agri-Food Canada and DSM Nutritional Products for their financial support of this project.

Furthermore I would like to acknowledge the financial support from the the Mexican government through the National Council on Science and Technology (CONACYT), the Secretariat of Public Education and the Mexican National Educational Workers Union (SNTE) that made my stay in Canada possible.

My gratitude is also extended to Jody Forslund for doing lots of paperwork for me and making my life easier.

I would like to acknowledge my friends and all the nice people I met during my stay in Canada who made this an unforgettable experience. Thanks to the Latin Club and my office mates Paul and Diana for your friendship and for making sure I did not forget Spanish.

Finally and most importantly I would like to thank my wife, my daughter, my parents, my brother, my niece and grandma for their love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ADG	Average daily gain
APEE	3-azidopropionic acid ethyl ester
BCM	Bromochloromethane
BES	Bromoethanesulfonate
BW	Body weight
CH_4	Methane
CO_2	Carbon dioxide
CoB	Coenzyme B
CoM	Coenzyme M
CP	Crude protein
DE	Digestible energy
DM	Dry matter
DMD	Dry matter disappearance
DMI	Dry matter intake
DNA	Deoxiribonucleic acid
EMPS	Efficiency of microbial protein synthesis
E3NP	Ethyl-3-nitrooxy propionate
GE	Gross energy
GHG	Greenhouse gas
Gt CO_2-eq	Gigatonnes of CO_2-equivalent

GWP_{100}	Global warming potential over a 100 year time span
H_2	Hydrogen gas
LAM	Liquid-associated microorganisms
MCR	Methyl-coenzyme M reductase
Methyl-CoM	Methyl-coenzyme M
ME	Metabolic energy
MGA	Melengesterol acetate
MH	Metabolic hydrogen
MHP	MH produced
MHR	MH recovered
MHR_{VFA}	MHR in the form of VFA
MHR_{CH4}	MHR in the form of CH_4
MHR_{H2}	MHR in the form of H_2
MON	Monensin
MPS	Microbial protein synthesis
NDF	Neutral detergent fibre
NH_3	Ammonia
NOP	3-nitrooxypropanol
OM	Organic matter
OMD	Organic matter disappearance
P + B + V	Sum of the proportion of propionate, butyrate and valerate
qPCR	Quantitative PCR
rRNA	Ribosomal ribonucleic acid

Rusitec	Rumen simulation technique
SAM	Solid-associated microorganisms
TMR	Total mixed ration
VFA	Volatile fatty acids

1. Literature Review

1.1. Introduction

Methane (CH_4) is an undesirable product of rumen fermentation because its production reduces the feed energy available for the animal and increases the carbon footprint of animal agriculture. Methane is a potent greenhouse gas (GHG) that contributes to global warming (Johnson and Johnson, 1995; IPCC, 2013). Various CH_4 mitigation strategies have been proposed, and among these the use of inhibitors represents one of the most studied options (Bauchop, 1967). Methane inhibitors have been shown to lower emissions in sheep, goats and beef cattle (Sawyer *et al*., 1974; Tomkins *et al*., 2009; Abecia *et al*., 2012) without adverse effects on animal productivity (McCrabb *et al*., 1997). Additionally, these inhibitors comprise a small group of CH_4 mitigation strategies and some of them have been successfully evaluated over long-term periods without signs of adaptation or negatively affecting dry matter (DM) intake (DMI) or body weight (BW) gain (Tomkins *et al*., 2009; Abecia et al., 2012; Hristov et al., 2015). However, negative side effects of the inhibitors on safety of animals, people, and the environment, together with undesirable physicochemical properties of some compounds have limited their use. The chemical structure of inhibitors such as bromochloromethane (BCM) and bromoethanesulfonate (BES) has been used as a model to develop new compounds suitable for animal use with high CH_4 mitigation potential. Investigations in this area resulted in the development of 3-nitrooxypropanol (NOP; Duval and Kindermann, 2012), a synthetic molecule similar in structure to methyl-coenzyme M (methyl-CoM) which reduces CH_4 production by quenching the active form of methyl-CoM reductase (MCR) (Prakash, 2014), the enzyme catalyzing the last step of methanogenesis (Thauer, 1998). At the time of initiating this thesis

research, no animal studies had been published to evaluate the use of NOP; however, limited *in vitro* work showed that NOP had the potential to completely inhibit methanogenesis.

Globally, beef cattle systems are the primary contributors to GHG emissions (mainly due to CH_4 from the animal and its manure, nitrous oxide from crop fertilization, and carbon dioxide (CO_2) from the use of fossil fuels and land use change) within the livestock sector. Additionally, beef cattle are the biggest source of CH_4 emissions both globally and in Canada (Environment Canada, 2015; Gerber *et al*., 2013). Thus, evaluating the effects of adding NOP to beef cattle diets on CH_4 production could have important implications for the beef industry both nationally and internationally.

1.2. Global methane emissions

The world's population is currently estimated at 7.3 billion people and it is expected to reach 9.7 billion people by 2050 (UN, 2015). This tremendous increase in population will undoubtedly increase the demand for food; in 2050 food production will need to be 60% greater than in 2005/2007 and as a result GHG emissions are expected to increase significantly (Alexandratos and Bruinsma, 2012). According to EPA (2012), global non-CO_2 anthropogenic emissions will increase from 11.4 gigatonnes of CO_2-equivalent (Gt CO_2-eq) in 2010 to 15.4 by 2030.

Global atmospheric mean concentrations of CO_2 (396 ppm), CH_4 (1,824 ppb) and N_2O (326 ppb) in 2013 increased by 42, 153 and 21%, respectively, compared to pre-industrial (i.e., before 1750) levels of 278 ppm for CO_2, 722 ppb for CH_4 and 270 ppb for N_2O (WMO, 2014). This increase is driven largely by the increase in the use of fossil fuels due to economic and population growth. It is extremely likely (95–100% confidence) that more than half of the

increase in global warming since the mid-20th century was caused by the anthropogenic increase in GHG concentrations (IPCC, 2014). The global warming potential over a 100 year time span (GWP_{100}) for CH_4 and N_2O is 28 and 265 times larger than CO_2 which has a GWP_{100} of 1 (Table 1.1).

Global anthropogenic GHG emissions in 2010 were estimated as 49 Gt CO_2-eq, which represents an annual increment of 2.03% from 1970 to 2010 considering that 27 Gt CO_2-eq were emitted in 1970 (IPCC, 2014). This may in fact be an underestimation as the GWP_{100} have lately been revised in particular for CH_4 (from 25 to 28). Using calculations based on the updated GWP_{100} values (IPCC, 2013), global anthropogenic GHG emissions for 2010 would be 52 Gt CO_2-eq. The most important GHG emitted by human activities on a CO_2-eq basis is CO_2, which contributes to 72% of the total, followed by CH_4 (20%), N_2O (5%) and fluorinated gases (2.2%) (IPCC, 2014).

Table 1.1. Annual emissions, atmospheric concentration, global warming potential over 100 year time spam (GWP_{100}), and lifetime for main greenhouse gases (Adapted from IPCC, 2013).

		Concentration[a]				
	Production, Gt CO_2-eq[b]	Year 1750	Year 2013	Increment, %	GWP_{100}	Lifetime, years
CO_2	37	278	396	42	1	Variable
CH_4	10	722	1824	153	28	12.4
N_2O	3	271	326	20	265	121

[a] CO_2, ppm; CH_4, ppb; N_2O, ppb.
[b] Production for year 2010.

Total global CH_4 emissions, including both natural and anthropogenic sources, were estimated at 14.15 Gt CO_2-eq (EPA, 2010). Projections of global anthropogenic CH_4 emissions for 2015 were estimated at 7.53 Gt CO_2-eq with the agriculture sector being the most important contributor accounting for 42.7% of the total (EPA, 2012). Within the agriculture sector, enteric fermentation accounted for 2.04 Gt CO_2-eq (EPA, 2012). Thus, based on previous data, enteric

CH_4 production contributes 64, 27 and 4% of the total CH_4 from agriculture, global anthropogenic CH_4, and global anthropogenic GHG emissions, respectively.

Gerber *et al*. (2013) utilized a life cycle assessment approach to identify the main sources of GHG coming from livestock production globally and determined that 7.1 Gt CO_2-eq were emitted per annum, which represents 14.5% of all anthropogenic emissions. Of the total GHG derived from the livestock sector, 44% corresponds to CH_4, 29% to N_2O, and 27% to CO_2 (Figure 1.1). Cattle account for 65% of global GHG livestock sector emissions (4.6 Gt CO_2-eq) with beef (2.5 Gt CO_2-eq) and dairy (2.1 Gt CO_2-eq) as the main contributors. Enteric fermentation releases CH_4, the largest single source of GHG emitted by livestock (2.8 Gt CO_2-eq) with cattle as the largest source of enteric CH_4.

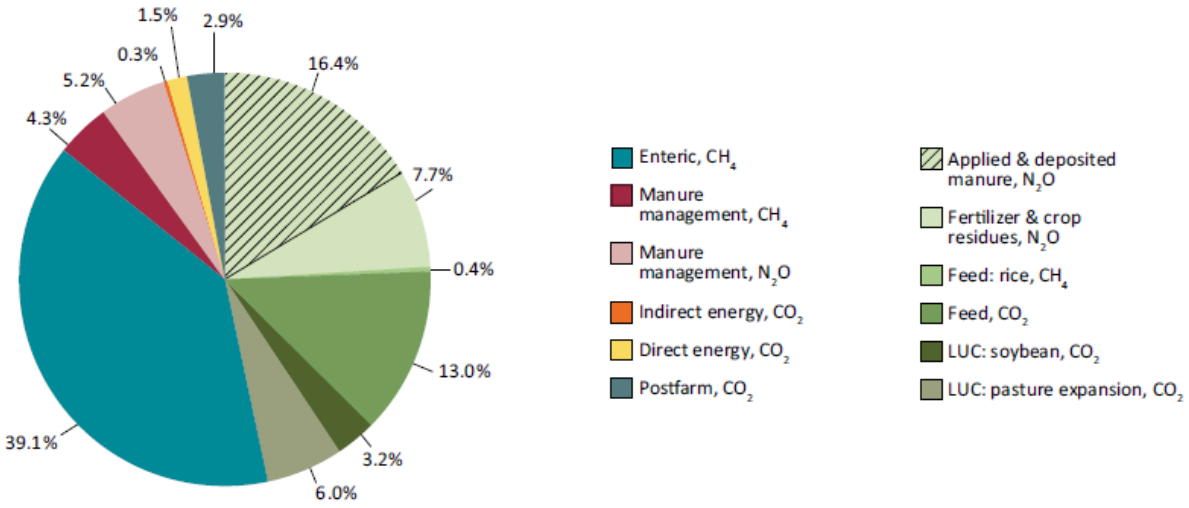

Figure 1.1. Global emissions from livestock supply chains by category of emissions (From Gerber *et al*., 2013).

While reduction of all GHG is desirable, special attention has been focused on lowering CH_4 emissions. This is because although CH_4 has a GWP_{100} 28 times greater than CO_2, its average lifetime in the atmosphere is only 12.4 yr, almost 10 times less than the lifetime of N_2O and much less than most fluorinated gases (IPCC, 2013). Thus, the benefits of reducing CH_4 emissions to the atmosphere in terms of global warming would be observed in a shorter period of time as compared to other gases.

1.2.1. Canada's greenhouse gases emissions

The most recent National Inventory Report (Environment Canada, 2015), estimated total GHG emissions for Canada at 726 Mt of CO_2-eq, 113 Mt (18%) above the 1990 total of 613 Mt. Canada's emissions represent about 1.5% of total global emissions. Land use, land use change and forestry estimates were excluded from the report. If included, these categories would have removed 15 Mt CO_2-eq which would have decreased total emissions by 2%. According to Environment Canada (2015), the energy sector including stationary combustion, transport and fugitive emissions accounted for 81.1% of Canada's emissions followed by agriculture (8.3%), industrial processes and product use (7.2%) and waste (3.4%) sectors. When 2013 emissions were broken down by GHG, the distribution of the emitted gases was as follows: CO_2 (78.5%), CH_4 (14.7%), N_2O (5.6%) and fluorinated gases (1.1%). Agriculture accounts for 27% and 70% of the national CH_4 and N_2O emissions, respectively. Methane produced by enteric fermentation (25 Mt CO_2-eq) is the main GHG that contributes to Canadian agriculture emissions, accounting for 41.7%. Thus, enteric fermentation from livestock accounts for 23% of anthropogenic CH_4 in Canada with over 95% of these emissions arising from cattle. Livestock emissions (enteric fermentation and manure management) represent 61% of agricultural emissions with beef being

the dominating source. Beef production released 26 Mt CO_2-eq in 2013 representing 70% of total GHG emissions from the livestock sector (Environment Canada, 2015).

1.3. Rumen methanogenesis

Methane is an end product of the microbial degradation of organic matter (OM) in aqueous anaerobic environments such as the digestive tract of animals (Thauer, 1998). The gastrointestinal tract of ruminants is an ideal habitat for gut microorganisms. The ruminant stomach is composed of four compartments (the rumen, reticulum, omasum and abomasum; Russell, 2002). The evolution of the reticulo-rumen make it possible for ruminants to eat and to retain fibrous material (rich in cellulose and hemicellulose) in the rumen for long periods (2–3 d), and to sustain a microbial population that digests such material in a symbiotic relationship with the host (Bannink and Tamminga, 2005). As a result of microbial fermentation, biomass that otherwise cannot be enzymatically digested by the host, is degraded (Bannink and Tamminga, 2005). Dietary carbohydrates (i.e., cellulose, hemicellulose, pectin, starch, fructans and soluble sugars) are the main fermentation substrates. They are degraded to their constituent hexoses and pentoses before being fermented to volatile fatty acids (VFA; mainly acetate, propionate and butyrate) via pyruvate (France and Dijkstra, 2005; Figure 1.2).

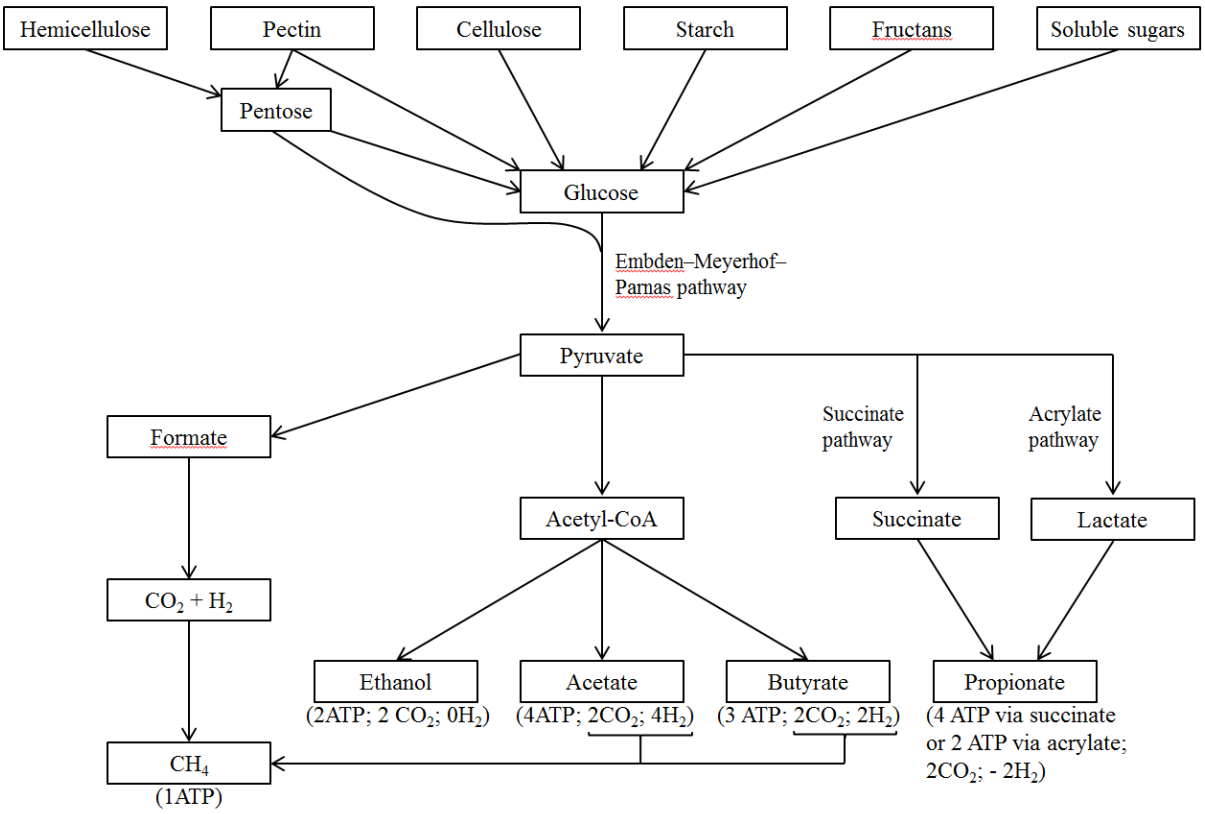

Figure 1.2. Major pathways of carbohydrate metabolism in the rumen (Adapted from France and Dijkstra, 2005 and Hristov *et al*., 2013a).

In addition to VFA, the process of carbohydrate digestion in the rumen yields CO_2 and MH (France and Dijkstra, 2005). Depending on the VFA formed, different amounts of MH are released (acetate and butyrate) or utilized (propionate). Minor VFA such as isobutyrate, valerate, isovalerate and caproate are also produced in the rumen, with combined molar proportions typically being less than 2%. Alcohols and lactate are also formed during fermentation, but its concentration is very low in the rumen, except when lactate accumulates causing rumen acidosis (Hristov *et al*., 2013a). Excess MH produced during fermentation is utilized during the formation of propionate and other products of fermentation such as valerate, caproate, lactate, ethanol and microbial biomass, and in the reduction of CO_2 to CH_4 (Ungerfeld, 2015). Formate can also be

used for CH_4 formation; however, most of it is first converted to H_2 and CO_2 (Russell, 2002). An overall equation of rumen fermentation and specific reactions for VFA and CH_4 formation is as follows:

57.5 ($C_6H_{12}O_6$) → 65 acetate + 20 propionate + 15 butyrate + 60 CO_2 + 35 CH_4 +25 H_2O

Source: Wolin, 1960.

$C_6H_{12}O_6 \rightarrow 2\ CH_3COCOOH + 2H_2$	(Pyruvate production)
$2\ CH_3COCOOH + 2H_2O \rightarrow 2\ CH_3COOH + 2CO_2 + 2H_2$	(Acetate production)
$2\ CH_3COCOOH + 4H_2 \rightarrow 2\ CH_3CH_2COOH + 2H_2O$	(Propionate production)
$2\ CH_3COOH + 2H_2 \rightarrow CH_3(CH_2)_2COOH + 2H_2O$	(Butyrate production)
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	(Methane production)

Source: Demeyer, 1991.

Changes in the molar proportion of rumen VFA are related to increases or decreases in the production of CH_4. For example, when ruminants consume a forage-based diet, the molar proportion of acetic, propionic, and butyric acids in the rumen are ≈ 60, 20 and 10%, respectively, whereas when ruminants consume a grain-based diet, molar proportions of these VFA are ≈ 40, 40 and 10%, respectively (Beauchemin and McGinn, 2005).The increase in molar proportion of propionate at the expense of acetate results in less CH_4 production (Beauchemin and McGinn, 2005).

The organisms mediating CH_4-forming reactions were originally thought to be bacteria, but are now recognized as belonging to a separate phylogenetic domain, the Archaea (Thauer, 1998). Archaea are prokaryotic microorganisms that belong to the third domain of life, distinct from bacteria and Eucarya (DasSarma *et al*., 2009). The main difference between bacteria and Archaea is that pseudomuereins are the basic constituents of the cell wall in Archaea in contrast to peptidoglycans for bacteria (DasSarma *et al*., 2009). Methanogenic Archaea or methanogens belong to the kingdom Euryarchaeota, comprised of 5 orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales, and Methanosarcinales), 10 families (Methanobacteriaceae, Methanocaldococcaceae, Methanococcaceae, Methanocorpusculaceae, Methanomicrobiaceae, Methanopyraceae, Methanosaetaceae, Methanosarcinaceae, Methanospirillaceae, and Methanothermaceae) and 31 genera (DasSarma *et al*., 2009). According to Janssen and Kirs (2008), few methanogens have been cultured from the rumen with an expectation that additional species will be identified and cultured in the future. Cultured species include *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanomicrobium mobile*, *Methanoculleu, Olentangyi*, *Methanosarcina spp*. *Methanobrevibacter smithii, Methanobrevibacter thaueri*, *Methanobacterium mobilis*, and *Methanobrevibacter wolinii* (Janssen and Kirs, 2008; Liu *et al*., 2008).

Methanobrevibacter is the most predominant methanogen found in the rumen belonging to the order of Methanobacteriales and often accounts for more than 50% of the methanogen population (St-Pierre, 2013). Most rumen archaea in the rumen consist of the genera Methanobrevibacter (62%), Methanomicrobium (15%), and rumen cluster C (16%), which is a group of uncultured rumen archaea (Janssen and Kirs, 2008).

Methanogens obtain energy for growth strictly from the reduction of CO_2 to CH_4 and are the only organisms in the rumen known to produce this gas (Thauer, 1998). These organisms are extremely sensitive to oxygen and therefore inhabit anaerobic environments (DasSarma *et al*., 2009). In the rumen, the substrates used by methanogens to produce CH_4 can be divided into acetate (acetoclastic pathway), H_2 and CO_2 (hydrogenothropic pathway), methyl, formate and/or other C_1 compounds (methylothropic pathway) (Figure 1.3; DasSarma *et al*., 2009; Thauer, 1998).

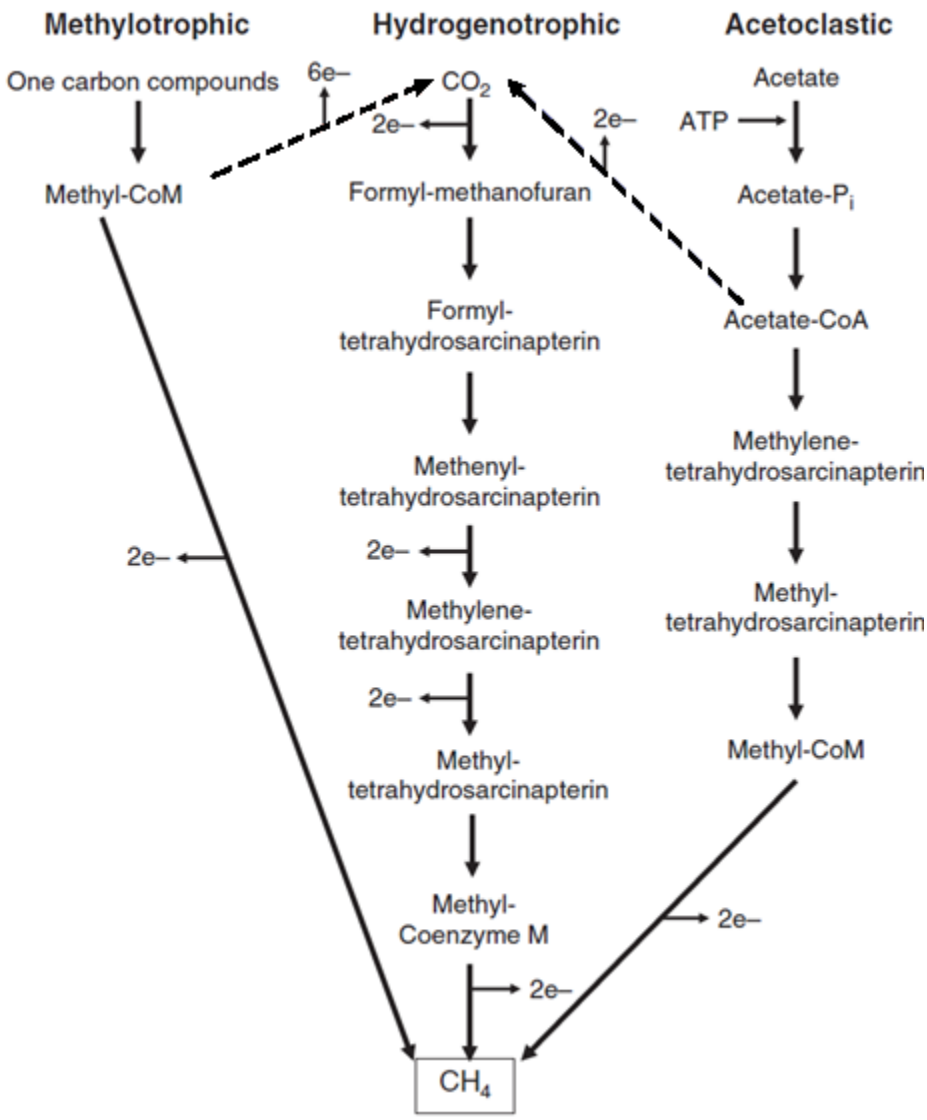

Figure 1.3. Pathways of methanogenesis. The dashed line from methyl-CoM to CO_2 indicates the ability of some Archaea to reverse the hydrogenotrophic pathway to produce CO_2, which can then move in the forward direction to produce CH_4. The dashed line from acetate-CoM to CO_2 indicates that CO, which is later converted into CO_2, is a by-product of the acetoclastic pathway (from DasSarma *et al*., 2009).

Most methanogens that exist inside of multicellular microorganisms utilize CO_2 and H_2 to form CH_4 (Saengkerdsub and Ricke, 2014). This also applies to the rumen, where 9 of the 11 clades of Archaea detected consist of H_2-utilizing methanogens (Janssen and Kirs, 2008). Unlike external environments, where products of biopolymer degradation accumulate, the digestive system of animals (rumen included) actively absorb fermentation products such as VFA, thereby avoiding continuous acumultion of end products. As a result, the hydrogenotrophic methanogens predominate over those that utilize acetate in these environments. The detailed hydrogenotrophic pathway (Figure 1.4) and step-by-step explanation was given by Saengkerdsub and Ricke (2014). Briefly, during the initial step of methanogenesis, a two electron reduction of CO_2 and methanofuran (MFR) are catalyzed by formyl-MFR dehydrogenase (1); subsequently, CO_2 is decreased to the formyl level. The formyl group is converted to formyl-tetrahydromethanopterin (H_4MPT) by formyl-MFR:H_4MPT formyltransferase (2). The cyclization of formyl-H_4MPT to methenyl-H_4MPT is catalyzed by N^5,N^{10}-methenyl-H_4MPT cyclohydrolase (3). The methenyl-H_4MPT is reduced in two steps (two electrons each) by the electron carrier coenzyme F_{420} to methylene-H_4MPT and methyl-H_4MPT by N^5,N^{10}-methylene-H_4MPT dehydrogenase (4) and N^5,N^{10}-methylene-H_4MPT reductase (5). The methyl group is subsequently transferred to coenzyme M (CoM) to form methyl-CoM by N^5-methyl H_4MPT:CoM-SH methyltransferase (6). Methyl-CoM is reduced to CH_4 by MCR (7) which uses coenzyme B (CoB) as an electron donor. The products of reaction are CH_4 and a disulfide of CoM and CoB that is referred to as heterodisulfide. Free CoM and CoB are regenerated by the reduction with H_2 by heterodisulfide reductase (8).

Methanogenic archaea that can reduce CO_2 with H_2 to CH_4 can be categorized in terms of the presence of cytochromes in their membranes. Methanogens in the order Methanosarcinales

contain cytochromes while methanogens in other orders do not (Thauer *et al*., 2008). The most important differences are the higher growth yields and H_2 threshold concentrations that are observed in methanogens with cytochromes (Thauer *et al*., 2008). However, it is accepted that CH_4 production from CO_2 and H_2 proceeds in the same manner for both types of methanogens, and only the reduction of the CoM‑S‑S -CoB complex with H_2 (Figure 1.4; reaction 8) differs between the two types (Thauer *et al*., 2008).

Methanogens can also be non-taxonomically classified into methanogens that are able to synthesize CoM intracellularly, such as *Methanobacterium mobile* or *Methanobrevibacter smithii*, and methanogens that cannot synthesize CoM, such as *Methanobrevibacter ruminantium* M1, that need to obtain external CoM from the medium (Balch and Wolfe, 1979). Coenzyme M is the smallest coenzyme ever decribed, which together with its methylated form, methyl-CoM, and the enzyme MCR are exclusively found in methanogenic Archaea (Thauer, 1998).

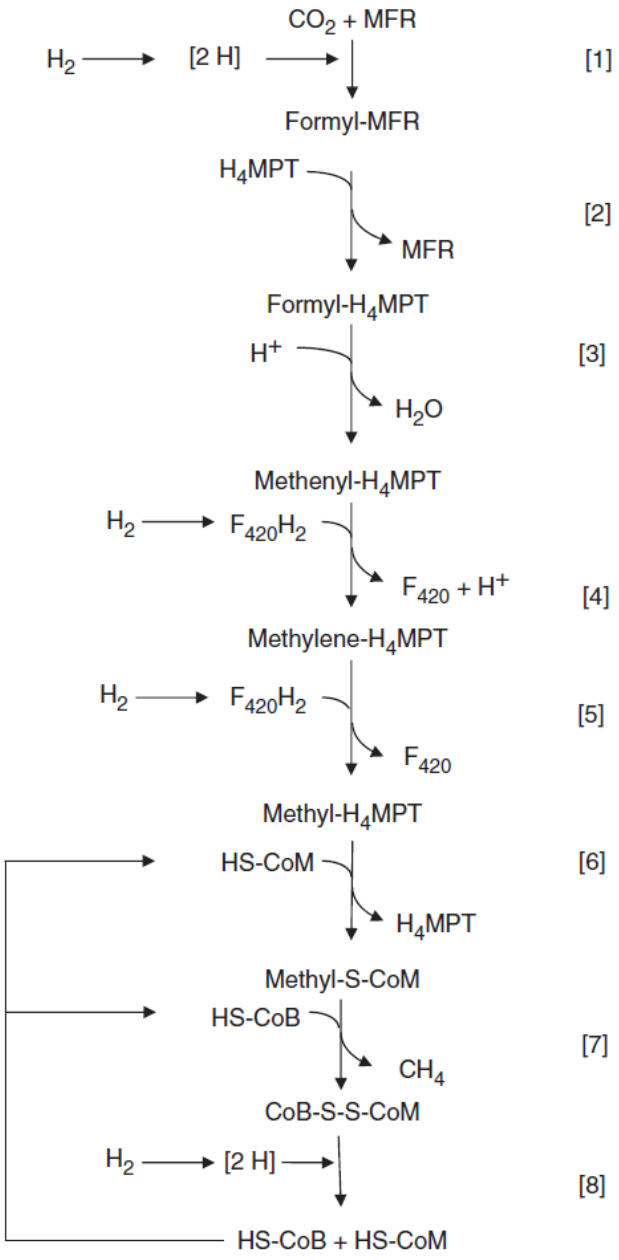

Figure 1.4. Pathway of methanogenesis from H_2 + CO_2. (1) formyl-MFR dehydrogenase, (2) formyl-MFR:H_4MPT formyltransferase, (3) N^5,N^{10}-methenyl-H_4MPT cyclohydrolase, (4) N^5,N^{10}-methylene-H_4MPT dehydrogenase, (5) N^5,N^{10}-methylene- H_4MPT reductase, (6) N^5-methyl-H_4MPT:CoM-SH methyltransferase, (7) MCR, and (8) heterodisulfide reductase (From Saengkerdsub and Ricke, 2014).

Glycolysis, and pyruvate oxidative decarboxylation to acetyl-CoA, is the first step in the formation of acetate and butyrate with both end products resulting in the release of H_2 (Ungerfeld, 2015). Hydrogen is a central metabolite in rumen fermentation and its partial pressure is an important determinant of rumen methanogenesis (Hegarty and Gerdes, 1999). Metabolic hydrogen (MH) for CH_4 formation occurs in three key states in the rumen, these being H_2, reduced cofactors (e.g., NADH and NADPH), and as free protons (H^+) (Hegarty and Gerdes, 1999). The regeneration of NAD^+ from NADH is regulated by the enzyme NADH ferredoxin oxidoreductase. Rumen H_2 is liberated by hydrogenase enzymes acting on reduced ferredoxin which allows reoxidation of cofactors. The activity of NADH ferredoxin oxidoreductase is primarily controlled by concentration of dissolved H_2 and suppressed by a high H_2 partial pressure (Hegarty and Gerdes, 1999). In the anoxic rumen environment, the re-oxidation of NADH to NAD^+ is driven by electron transfer to acceptors other than oxygen, with CO_2 as the main sink for CH_4 formation (McAllister and Newbold, 2008).

Metabolic hydrogen not used for CH_4 production is also redirected to propionate and butyrate production (butyrate production utilizes MH although this process results in net MH production), with only a small proportion being directed to atypical sinks like H_2 (Ungerferld, 2015). A production of 100 L/d of H_2 may be estimated in the rumen of a sheep producing 25 L/d of CH_4 considering that 4 L of H_2 are required per litre of CH_4 produced (Hegarty and Gerdes, 1999). Because methanogens efficiently use H_2, it does not accumulate in the rumen (McAllister and Newbold, 2008). The normally low concentrations of H_2 in the rumen headspace (less than 1%) reflects the efficient utilisation of H_2 by methanogens in the rumen (Janssen, 2010). However, despite the low H_2 concentration, hydrogen in all its forms plays an important role in regulating rumen fermentation (Hegarty and Gerdes, 1999). Accumulation of H_2 is

energetically inefficient as it inhibits hydrogenase activity and limits the oxidation of carbohydrates when alternative pathways for the disposal of H_2 are absent (McAllister and Newbold, 2008).

The balance of H^+ and dissolved H_2 concentrations directly determines the redox potential of the rumen and the products of fermentation. Additionally, the H_2 partial pressure has substantial effects on the energetics of methanogens (Hegarty and Gerdes, 1999). It is generally considered that 1 mol of ATP would result from the production of 1 mol of CH_4. However, an increased partial pressure of H_2 may cause ATP yield to reach 2 mol ATP/mol CH_4, contrary to the 0.33 mol ATP/mol CH_4 obtained when H_2 partial pressure is critically low (Keltjens and Vogels 1996).

Hydrogen gas is non-polar and poorly soluble in water. Thus, it is assumed to pass freely through microbial membranes, contrary to H^+ concentration in the cytoplasm which is more strictly regulated by microbial cells (Hegarty and Gerdes, 1999). Information on the concentration of dissolved H_2 concentrations in the rumen is scarce. Analytical methods for dissolved H_2 are relatively insensitive for measuring the typically low concentrations of dissolved H_2 that occur in the rumen and it is sometimes reported as zero (Robinson *et al*., 1981; Janssen, 2010). The dissolved H_2 can be estimated based on the H_2 partial pressure in the rumen gas phase, and the maximum H_2 solubility at 39°C (737 μM), assuming a maximum possible 100% concentration of H_2 in the rumen headspace (Hegarty and Gerdes, 1999; Janssen, 2010). Dissolved H_2 concentrations in rumen liquid can vary over a range of 0.1–250 μM (Hegarty and Gerdes, 1999; Janssen, 2010).

Despite a paucity of data, dissolved H_2 concentrations in the rumen are thought to be greater in ruminants fed readily digestible feed compared with those fed low quality feed,

particularly after feeding (Janssen, 2010). Elevated dissolved H_2 concentrations are required for methanogens to growth fast enough to prevent washout from the rumen at high ruminal passage rates, at suboptimal ruminal pH values, or in the presence of inhibitors. In contrast, lower dissolved H_2 concentrations are required when the passage rate is lower, when the pH is near optimum, or when methanogens are not affected by inhibitors (Janssen, 2010).

The model proposed by Janssen (2010) summarizes the effects of prevailing H_2 concentration in the rumen on the thermodynamics of fermentation. Briefly, fermentation pathways producing H_2 (e.g., acetate production) are favoured at low dissolved H_2 concentrations, while H_2 utilizing pathways (e.g., propionate production) are favoured when dissolved H_2 concentrations are high. Therefore, feeds and conditions that result in low dissolved H_2 concentration would result in more H_2 formation, less propionate formation, and more CH_4 formation per mole of glucose fermented. Conversely, conditions that favour high dissolved H_2 concentrations would result in less H_2 formation per mole of glucose, less production of CH_4 and more propionate.

Methanogens, which typically account for approximately 1% of total microbial community in the rumen (Ziemer *et al*., 2000; Belanche *et al*., 2014), exist synergistically with bacteria and symbiotically with rumen ciliate protozoa and fungi (Cersosimo and Wright, 2015). There is a syntrophic interaction (the combined effect of two organisms in completing a chemical reaction) among bacteria, protozoa, fungi and methanogens that involves interspecies H_2 transfer (Leng, 2014). Interspecies H_2 transfer refers to the transfer of H_2 from fermenting organisms to methanogenic Archaea (Leng, 2014). This process mantains a low partial pressure of H_2 in the vicinity of actively fermenting organisms (Leng, 2014). According to Janssen and Kirs (2008), methanogens are found free in the rumen fluid, attached to feed and rumen epithelia, and

associated to ciliated protozoa extracellularly (Vogels *et al*., 1980) and intracellularly (Finlay *et al*., 1994). Methanogens are known to carry out interspecies H_2 transfer with other rumen microorganisms, especially with rumen protozoa (Hook *et al*., 2010). The close physical association between methanogens and protozoa is advantageous for H_2 transfer because the rate of CH_4 production is greater when microbes that use H_2 are closer to those that produce it (de Bok *et al*., 2004). Rumen protozoa benefit from the removal of H_2 that can inhibit their metabolism, while methanogens get energy by reducing CO_2 with H_2 to form CH_4 (Cersosimo and Wright, 2015). It was estimated that protozoa-associated methanogens can account for between 9 and 37% of total CH_4 production in the rumen (Finlay *et al*., 1994; Newbold *et al*., 1995).

1.4. Strategies to reduce enteric methane emissions

Cattle typically lose 2 to 12% of their ingested energy as CH_4 (Johnson and Johnson, 1995). This variation reflects an opportunity for reducing enteric CH_4 emissions from ruminants. A range of strategies have been proposed as enteric CH_4 mitigation options, and these have been reviewed extensively by others (Beauchemin *et al*., 2008; Hook *et al*., 2010; Hristov *et al*., 2013b; Knapp *et al*., 2014). Some of these are briefly discussed below, with greater detail provided on the use of chemical inhibitors because this is the mitigation strategy pursued in my thesis research.

1.4.1. Monensin

Ionophores such as monensin (MON) are a class of antibiotics routinely fed to beef cattle in feedlots and more recently to dairy cattle (Russell and Houlihan, 2003). Monensin reduces

CH_4 emissions by inhibiting H_2 producers in the rumen (gram positive bacteria and protozoa), and redirecting MH to propionate (Russell and Houlihan, 2003). Monensin can also improve feed efficiency, N metabolism, prevent bloat, and prevent ketosis post-calving (Grainger *et al*., 2008). Moreover, MON supplementation has been reported to moderately reduce (<10%) enteric CH_4 emissions from cattle in a number of experiments (Sauer *et al.*, 1998; McGinn *et al*., 2004; Odongo *et al*., 2007). Appuhamy *et al*. (2013) conducted a meta-analysis to quantitatively summarize the effect of MON on CH_4 production. They reported that MON decreased DMI in dairy and beef cattle, and that CH_4 production in these animals was decreased by 19 and 6 g/d, respectively. Monensin effects on CH_4 production are dose dependant and CH_4 (g/kg DMI) appears to be decreased only when doses > 24 ppm are included in the diet (Beauchemin *et al*., 2008), although adaptation may occur overtime (Guan *et al*., 2006). The reduction in CH_4 production observed with MON supplementation is also due to the reduction in feed intake (Johnson and Johnson, 1995). Currently, MON is recommended as a mitigation practice through its effect on feed efficiency, particularly when high grain or mixed grain-forage diets are used (Hristov *et al*., 2013a). However, the tendency to reduce the use of antimicrobials in animal production have limited its use in regions of the world such as the European Union, thus it is not a long-term solution (Beauchemin *et al*., 2008).

1.4.2. Plant extracts

Plant extracts such as tannins and saponins are natural alternatives to chemical CH_4 inhibitors, which can be negatively perceived by consumers. However, most experiments with plant extracts have been done *in vitro* and the effects on CH_4 production are highly variable (Martin *et al*., 2010).

Tannins are polyphenolic substances (Goel and Makkar, 2012) that reduce CH_4 production by direct inhibition of methanogens and indirect reduction of H_2 production (Tavendale *et al*., 2005). The source for tannins is often tropical shrub legumes (Beauchemin *et al*., 2008). Tannins can be classified as either being condensed or soluble; however, most research has focused on condensed tannins because their lower risk of toxicity (Beauchemin *et al*., 2008). Animal supplementation with condensed tannins has been reported to have no effect on CH_4 production (Beauchemin *et al*., 2007) or to reduce CH_4 production at the expense of digestibility (Grainger *et al*., 2009). Although tannin supplementation has been reported to strongly inhibit CH_4 reduction (50-58%; g/kg DM) in some *in vivo* experiments (Puchala *et al*., 2005; Animut *et al*., 2008), the substantial decrease in CH_4 reduction is difficult to achieve without compromising animal production (Goel and Makkar, 2012).

Saponins are natural detergents that indirectly reduce CH_4 production by suppressing ruminal protozoa, selectively inhibiting some bacteria, and/or reducing methanogens (Goel and Makkar, 2012; Patra, 2012). A limited number of *in vivo* experiments, mainly in sheep, have been conducted to evaluate the potential of saponins to reduce CH_4 emissions. Different sources and concentrations in the diet may affect their effectiveness. When *Yucca schidigera* extract was fed to sheep, Śliwiński *et al*. (2002) did not observe effects on CH_4 production, while Santoso *et al*. (2004) and Wang *et al*. (2009) reported a reduction in CH_4 emissions with no effect on digestibility. When Holtshausen *et al*. (2009) supplemented saponin-containing *Yucca schidigera* and *Quillaja saponaria* to dairy cattle, no effects on CH_4 production were observed. Results from a meta-analysis conducted by Jayanegara *et al*. (2014) indicate that a low dose (≤5 g/kg DM) of saponin decreased CH_4 emissions by 8.6% without negatively affecting digestibility. While a higher dose (>5 g/kg DM) did not result in a further reduction in CH_4

production (7.3%), nutrient digestibility was decreased. The use of plant extracts has potential to reduce CH_4 emissions, but more *in vivo* experiments must be conducted before recommending them for use in commercial ruminant production systems.

1.4.3. Increased concentrate feeding

Increasing the proportion of concentrate in the diet increases the rates of ruminal fermentation and passage from the rumen, and lowers ruminal pH, which favours development of starch-fermenting microbes with consequent shift of VFA production from acetate to propionate (Johnson and Johnson, 1995; Hook *et al*., 2010; Martin *et al*., 2010). Methane production is decreased because the relative proportion of ruminal MH sources declines while that of MH sinks increases (Martin *et al*., 2010). In dairy cattle, increasing the use of concentrates decreased CH_4 production by 2% for every 1% increase of non-fibre carbohydrate in the diet with a maximum reduction of approximately 15% (Knapp, 2014). The use of concentrates in dairy cattle diets to reduce CH_4 emissions should not exceed ~50% of the dietary DM to avoid negative effects on milk fat content (Beauchemin *et al*., 2008). In beef cattle increasing concentrate proportion in the diet from 35 to 60% decreased total CH_4, CH_4 per kg of DMI and as % of gross energy (GE) intake by 30, 4, and 9% respectively (Lovett *et al*., 2003). However, when concentrate was increased to 90% of the diet DM, the same CH_4 variables were decreased by 18, 33, and 27%, respectively. A meta-analysis by Sauvant and Giger-Reverdin (2009) showed that the relationship between CH_4 and concentrate proportion in the diet is not linear and that reductions in CH_4 occur only when concentrate in the diet is >35-45%, depending upon the level of DMI. Increasing the proportion of concentrate in the diet can lower CH_4 emissions per unit of feed intake and animal product if production remains the same or is

increased (Hristov *et al*., 2013b). However, including more concentrate in animal diets may increase total GHG emissions as more grain must be grown, processed and transported (Beauchemin *et al*., 2008). Furthermore, concentrate use may be difficult to implement in many parts of the world because it may not be economically feasible and/or socially acceptable (Beauchemin *et al*., 2008; Hristov *et al*., 2013a).

1.4.4. Lipid supplementation

Lipid sources are routinely used in animal production to increase energy content of the diet. The sources can be animal origin, such as tallow, or plant origin such as extracted plant oils, oilseeds and high fat byproducts (Grainger and Beauchemin, 2011). Addition of unprotected lipids to the diet of ruminants effectively reduces CH_4 emissions (Beauchemin et al., 2008). The reduction occurs due to various mechanisms including the reduction of OM fermentation, activity of methanogens and protozoal numbers, reduction in DM intake, and by redirecting H_2 towards the biohydrogenation of unsaturated fatty acids (Johnson and Johnson, 1995; Beauchemin, *et al*., 2008). Patra (2013) conducted a meta-analysis using a database that included 105 treatment means obtained from 1339 observations of dairy and beef cattle in 29 experiments. The study reported a maximum crude fat concentration of 6% in the diet may reduce CH_4 emissions by 15% and at the same time improve milk production when compared to a diet containing 2% fat. Total crude fat content of the diet should not exceed 6% otherwise a depression in DMI with consequent depression in milk production or BW gain may occur (Beauchemin *et al*., 2008; Patra, 2013).

As demonstrated *in vitro* (Dohme *et al*., 2001), individual fatty acids differ in their CH_4 mitigation potential. Fats with high concentrations of C12:0, C18:3 and polyunsaturated fatty

acids are associated with greater decrease in CH_4 production (Patra, 2013). Similarly to the use of concentrates, the useof supplemental lipids as a strategy to reduce CH_4 is cost dependent and needs to result in an economical net reduction in GHG emissions before it can be recommended as a CH_4 mitigation strategy (Beauchemin *et al*., 2008).

1.4.5. Electron acceptors

Compounds such as sulfate and fumarate added to the diet reduce CH_4 production in short term experiments (Bayaru *et al*., 2000; Wood *et al*., 2009; van Zijderveld *et al*., 2010). Nitrate is the most studied electron acceptor and has shown promising results for CH_4 reduction (Nolan *et al*., 2010; van Zijderveld *et al*., 2010; Lee *et al*., 2015; Hulshof *et al*., 2015); however, issues such as excessive production of ruminal ammonia (NH_3) and toxicity from intermediate products (nitrite) may limit the use of nitrate (Hristov *et al*., 2013b). Nitrate is the only electron acceptor that has decreased CH_4 on a long-term basis when supplemented to dairy cows (van Zijderveld *et al*., 2011) and sheep (Li *et al*., 2012). Nitrate showed consistent efficacy to reduce CH_4 emissions from eight studies and 25 treatments (Lee and Beauchemin, 2014). Methane emissions were decreased linearly with increasing levels of nitrate, and a reduction of 8.3 g of CH_4/ kg of DMI is expected per each g of nitrate supplied per kg of BW (Lee and Beauchemin, 2014). Additionally, the combination of nitrate with other mitigation strategies such as sulfate (van Zijderveld *et al*., 2010) and linseed oil (Guyader *et al*., 2015) have been shown to be additive in terms of reducing CH_4 emissions. Nitrate is also a source of non-protein N and can be used to partially replace urea in ruminant diets. However, to avoid toxicity, animals need to be gradually acclimatized to nitrate consumption, a requirement that may prove challenging under commercial production conditions (Lee and Beauchemin, 2014).

1.4.6. Inhibitors

The use of enteric CH_4 inhibitors has been widely studied from the standpoint of energy use efficiency of ruminants and more recently from the standpoint of reducing enteric CH_4 production. Based on chemical structure, it is possible to divide the specific inhibitors of methanogenesis into two general categories: 1) methane analogues, such as chloroform or BCM, and 2) CoM analogues, such as BES and NOP. Compounds with a more complex structure, such as cyclodextrin, have also been reported to inhibit methanogenesis.

The study of inhibitors of methanogenesis originated by serendipity during an *in vitro* experiment focused on CH_4 production from bovine rumen contents (Bauchop, 1967). Excessive foam production during fermentation was decreased with an antifoam spray, but subsequent gas analysis showed that CH_4 was no longer present in the gas mixture and H_2 accumulated instead. The composition of the antifoam spray was unknown, but had a chloroform-like odor (Bauchop, 1967). The chloroform-like odor and the structural analogy between CH_4 and chloroform supported the idea that chloroform was present in the antifoam spray and that it was responsible for CH_4 reduction. To test this hypothesis, a series of *in vitro* experiments were conducted to evaluate the antimethanogenic effects of chloroform and other CH_4 analogues such as dichloromethane and tetrachloromethane (Bauchop, 1967). All the evaluated compounds decreased CH_4 production by 86 to 100% and this was accompanied by H_2 accumulation in different amounts but less than that expected from the decrease in CH_4 production (Bauchop, 1967). Subsequently, Wood *et al*. (1968) discovered that low concentrations of CH_4 analogues inhibit cobamide-dependent methyl-transfer reactions by forming complexes with reduced vitamin B_{12} (Chalupa, 1977).

Table 1.2. Enteric methane inhibitors evaluated *in vivo*.

Inhibitor	Specie	Dose	Mode of supplementation	Duration, days	CH_4 reduction, %	H_2	Intake	Animal production	Methanogens
Bromochloromethane	Beef[(1)]	5.5 g	Added to feed	28	100[a]	NE	NA	NA[d]	NE
	Goats[(2)]	0.3 g/100 kg BW	Added to feed	70	33	NE	NA	NA[d,e]	NA
	Goats[(3)]	4 g/100 kg BW	Mixed with feed	33	91	↑	NA	NE	↓
	Beef[(4)]	0.3 g/100 kg BW	Added to feed	90	60	NE	NA	NA[d]	NE
Chloroform	Dairy[(5)]	1.5 mL	Dosed into the rumen	42	94-95[b]	NE	NA	NA[d]	↓
Chloral hydrate	Sheep[(6)]	4 g	Dosed into the rumen	5	100	↑	NE	NE	NE
Bromoethanesulfonate	Sheep[(7)]	2 g	Dosed into the rumen	7	99[c]	↑	↓	NE	NE
3-Nitrooxypropanol	Sheep[(8)]	0.1	Dosed into the rumen	30	25	NE	NA	NA[d]	NA
	Dairy[(9)]	0.5	Dosed into the rumen	35	5	NE	NA	NA[d,e]	NE
	Dairy[(9)]	2.5	Dosed into the rumen	35	7	NE	NA	NA[d,e]	NE
	Dairy[(10)]	2	Mixed with feed	28	60	NE	NA	↑[d], NA[e]	↓
	Dairy[(11)]	40 mg/kg DMI	Mixed with feed	84	26	↑	NA	↑[d], NA[e]	NE
	Dairy[(11)]	60 mg/kg DMI	Mixed with feed	84	31	↑	NA	↑[d], NA[e]	NE
	Dairy[(11)]	80 mg/kg DMI	Mixed with feed	84	32	↑	NA	↑[d], NA[e]	NE

BW, bodyweight; DMI, dry matter intake; NA, not affected; NE, not evaluated, ↑, increased; ↓, decreased.
[(1)] Johnson *et al*., 1972; [(2)] Abecia *et al*., 2012; [(3)] Mitsumori *et al*., 2012; [(4)] Tomkins *et al*., 2009; [(5)] Knight *et al*., 2011; [(6)] Van Nevel *et al*., 1969; [(7)] Immig *et al*., 1996; [(8)] Martínez-Fernández *et al*., 2014; [(9)] Reynolds *et al*., 2014; [(10)] Haisan *et al*., 2014; [(11)] Hristov *et al*., 2015.

[a] CH_4 was completely inhibited from 3-6 h post-feeding, with gradual recovery up to 15 h post-feeding.

[b] 94-95% decrease on d 4-5. Production slowly recovered to ~62% of pre-treatment levels by d 39.

[c] On d 4, CH_4 concentration approached control values. On d 7 CH_4 concentration was completely restored.

[d] Body weight gain

[e] Milk production

Since then, a series of experiments were conducted both *in vitro* and *in vivo* to evaluate the CH_4 inhibitory potential of many different compounds. For instance, Van Nevel *et al*. (1969) reported that CH_4 production was completely inhibited in sheep when 4 g of chloral hydrate were infused directly into the rumen cannula with a further decrease in acetate and an increase in propionate concentration, together with H_2 accumulation in the rumen. Trei *et al*. (1971) showed that simple halomethanes such as BCM were potent inhibitors of methanogenesis. This was confirmed *in vivo* by Johnson *et al*. (1972), who administered 5.5 g of BCM to steers and reported complete inhibition of methanogenesis for 3-6 h after feeding and a gradual recovery up to 15 h post feeding to pre-feeding levels. Methane reduction was accompanied by increased propionate and decreased acetate concentration in the rumen (Johnson *et al*., 1972).

Because halomethanes are volatile liquids and therefore not suitable feed additives, some researcherss focused on the development of new CH_4 inhibitors with more desirable physical properties (Czerkawski and Breckenridge, 1975a). However, these inhibitors with improved characteristics were not very effective in reducing CH_4 production *in vivo* (Czerkawski and Breckenridge, 1975b). The study of CH_4 analogues has continued to date, with chloroform and BCM as the most successful compounds within this category tested *in vivo*. Knight *et al*. (2011), using dairy cows, evaluated the effect of extended infusion of chloroform (1.5 mL/d) through the rumen cannula. They reported a 94-95% reduction in CH_4 emission within 4-5 d; however, CH_4 production slowly recovered to ~62% of pre-treatment levels by d 39. Some *in vivo* studies evaluating BCM have also been published in the past decade confirming its antimethanogenic effect in goats (Abecia *et al*., 2012; Mitsumori *et al*., 2012) and reporting long-term CH_4 reduction in beef (Tomkins *et al*., 2009). Chloroform can cause liver injuries and skin cancer (Golden *et al*., 1997; Plaa, 2000) whereas BCM is an ozone depleting compound that has been

banned in some countries (Tomkins *et al*., 2009). Thus, most recent studies with chloroform (Knight *et al*., 2011) and BCM (Tomkins *et al*., 2009; Abecia *et al*., 2012; Mitsumori *et al*., 2012) were conducted without the aim of validating these compounds as potential CH_4 mitigation strategies at the farm level, but to better understand the metabolism in the rumen when methanogenesis is inhibited and investigate possible effects on animal performance. Although previous CH_4 inhibitors cannot be recommended for commercial use for CH_4 mitigation, their chemical structure has been used as a model for the development of new CH_4 inhibitors that are not harmful to people, animals or the environment.

The study of inhibitors corresponding to methyl-CoM analogues, originated in the 70's after the discovery of CoM, a cofactor related to methyl transfer in methanogenesis (McBride and Wolfe, 1971). The methylated forms of CoM and CoB are utilized as substrates by MCR in the last step of methanogenesis to produce CH_4 (Thauer, 1998). Once CoM structure was revealed to be 2-mercaptoethanesulfonate (Taylor and Wolfe, 1974), experiments were conducted to evaluate the CH_4 inhibitory effects of diverse CoM structural analogues. For instance, Gunsalus *et al*. (1978) reported that BES was the most potent inhibitor among 22 compounds evaluated *in vitro*, with a concentration of 7.9 μM reducing CH_4 production by 50%, followed by 2-chloroethanesulfonate, a less potent inhibitor that required a 9-fold greater concentration to cause a similar inhibition. When evaluated in sheep (Immig *et al*., 1996), BES infusion through the rumen canula resulted in drastic decrease of CH_4 concentration to nearly zero, decreased acetate, and increased propionate and H_2 concentrations. Additionally, MH recovery was decreased by 30%, suggesting accumulation of non-determined reduced end products such as formate. However, after 7 d of BES infusion, CH_4 and VFA concentrations were restored to control values, thus adaptation was evident (Immig *et al*., 1996). Further

experiments evaluating BES were mainly done *in vitro* to further answer research questions about methyl-CoM, MCR, methanogens, and to better understand how the compound inhibited rumen fermentation (Nollet *et al*., 1997; Lopez *et al*., 1999; Ungerfeld *et al*., 2004; Zhou *et al*., 2011). However, the use of BES as a strategy to reduce methanogenesis at the farm level was abandoned since CH_4 concentration was increased to control values.

Bromoethanesulfonate has been used as a model to develop compounds with similar characteristics that can reduce CH_4 production on a long term basis. Soliva *et al*. (2011) reported the effects of pure compounds on CH_4 production, including 3-azido-propionic acid ethyl ester, a BES analog. 3-azido-propionic acid ethyl ester decreased CH_4 production by 98% compared to the control treatment and was the most effective inhibitor among 7 natural and synthetic compounds evaluated.

The disclosure of the crystal structure of MCR by Ermler *et al*. (1997), together with the availability of in silico techniques and virtual screening, has provided new opportunities to development of compounds that can compete for the active site in MCR reserved for methyl-CoM. Investigations in this area by DSM Nutritional Products (Basel, Switzerland) led to the recent development of a synthetic molecule, NOP (Duval and Kindermann, 2012). The enzyme MCR possesses a prostetic group (F_{430}) composed of two moles of a nickel porphinoid, which can traverse the oxidation states I, II, and III of Ni, but has to be in the Ni(I) form to be active (Thauer,1998). 3-Nitrooxypropanol is a structural analog of methyl-CoM with the ability to quench the active form of MCR and inhibit methanogenesis (Prakash, 2014). 3-nitrooxypropanol was selected based on a screening process via computer simulation of thousands of compounds with the objective of identifying those with the potential ability to inhibit MCR. A reduced number of compounds was selected for synthesis and evaluation *in vitro*. The best two were

ethyl-3-nitrooxy propionate (E3NP) and NOP, which were subsequently evaluated using the batch culture technique and in sheep (Martínez-Fernández *et al*., 2014). The *in vitro* study included the well-known CH_4 inhibitor BCM as a positive control (160 and 320 μmol/L), E3NP (25 and 50 μmol/L) and NOP (33 and 66 μmol/L). Methane production was decreased by 86 to 96% with the evaluated inhibitors as compared to the control with no significant differences among inhibitors (Martínez-Fernández *et al*., 2014). The study with sheep included a control treatment with no inhibitor, E3NP (100 mg/d) and NOP (100 mg/d). The CH_4 production was decreased by 15 and 26% on d 14, and by 22 and 24% on d 29-30 with E3NP and NOP, respectively. No negative effects were reported for BW or DMI with either inhibitor; however, CH_4 reduction was accompanied by decreased molar proportion of acetate and increased molar proportion of propionate with both additives.

Based on those studies, NOP was selected for further evaluation in cattle and various studies were conducted in Canada, the United Kingdom and more recently in the United States to assess the effectiveness of this compound for dairy and beef cattle. The present literature review on NOP includes only experiments conducted in dairy cattle, as the only beef studies conducted using NOP are presented within the research chapters of my thesis.

Reynolds *et al*. (2014) evaluated different doses of NOP (0, 0.5 and 2.5 g/d) into the rumen using lactating dairy cows. Addition of NOP decreased CH_4 production by 4.4 and 6.7% with the low and high dose, respectively without affecting DMI, digestibility or milk yield, but slightly increased milk protein (5%) and casein (6%) with 2.5 g/d of NOP. These authors observed a potent but transient reduction in CH_4 production 1 to 2 h after feeding, which was thought to be due to the mode of NOP was dosed (directly into the rumen cannula), suggesting that mixing NOP with the diet may be a more effective delivery method (Reynolds *et al*., 2014).

The reduction in CH_4 was accompanied by reduction in total methanogens, total VFA and the molar proportion of acetate, while propionate increased with 2.5 g/d of NOP (Reynolds *et al*., (2014).

When NOP (2.5g/d) was fed to lactating dairy cows by Haisan *et al*. (2014), CH_4 production was decreased by 60% with no effect on DMI, milk production or milk composition with a reduction in total methanogens.The large CH_4 reduction observed in this experiment was attributed to the mode of providing NOP to the cows (i.e., mixed with the feed), which allowed them to consume NOP at a consistent rate over the day as compared with a single dose of NOP directly into the rumen (Haisan *et al*., 2014). In this study, NOP decreased acetate and tended to increase propionate proportion. Interestingly, cows consuming NOP gained more BW, presumably because the reduction in CH_4 emissions increased the energy available for bodyweight gain. Cows used I the experiment were past peak lactation, thus the increase in BW as opposed to milk yield, was attributed to differences in portioning of metabolic energy (ME) intake as ME directed to milk production decreases while that towards body tissue increases as days in milk increase (Haisan *et al*., 2014).

Recently, Hristov *et al*. (2015) observed an average reduction in CH_4 production of 30% over 12 weeks, when NOP was added to the diet of lactating dairy cows at 40, 60 and 80 mg NOP/kg DM with no adaptation to the compound. Feed intake, milk production, and fibre digestibility were not affected by NOP but milk protein and lactose yields were increased. Confirming the observations of Haisan *et al*. (2014), Hristov *et al*. (2015) reported an average increase in BW of 80% for all NOP treatments compared with the control, with the medium dose (60 mg NOP/kg DM) showing up to a 114% increase, suggesting a partial redirection of energy from CH_4 to tissue deposition for cows receiving NOP. Additionally, that study reported effects

of NOP on H_2. As expected based on studies with inhibitors such as BCM and BES, NOP addition to the diet of dairy cows resulted in 64-fold increase in H_2 production for the high dose (from 0.02 to 1.27 g/d for 0 and 80 mg NOP/kg DM, respectively). Interestingly, H_2 production declined after 6 weeks of NOP addition.The authors suggested that the increase in H_2 emissions, along with the lack of effect on fibre digestibility or feed intake, may be indicative of the capacity of the rumen to adapt to elevated H_2 concentrations. According to Hristov *et al*. (2015), H_2 production from cows receiving NOP (80 mg/kg DM) represents a small proportion (3%) of H_2 expected from the decrease in CH_4 production, suggesting H_2 redirection to alternative sinks like formate, microbial biomass or reductive acetogenesis .

1.5. Summary

Methane production is an important component of global GHG emissions. The agriculture sector is the primary contributor to global anthropogenic CH_4 production with enteric fermentation from livestock as the main source. The benefits of reducing CH_4 production in terms of global warming would be observed earlier as compared to other GHG due to it shorter life span in the atmosphere and relatively large GWP_{100}. Ruminants emit CH_4 as a byproduct of microbial fermentation of feed in in the rumen. Methanogens utilize H_2 and CO_2 produced during fermentation for CH_4.formation This process represents a loss of energy to the animal. Several strategies have been evaluated based on their potential to reduce CH_4 production, including the use of enzymatic inhibitors of methanogenesis. Chloroform, BCM or BES, have long been recognized for their ability to inhibit CH_4 production; however, these compounds have not been used to mitigate CH4 emissions on-farm because of toxicity, environmental concerns or because the observed decrease in CH_4 production was not sustained overtime. Research in this area has

focused on identifying and developing inhibitors with improved characteristics that reduce methanogenesis without adverse effects on humans, animals or the environment. 3-Nitroxypropanol is a novel inhibitor that decreased CH_4 production in sheep and more recently in dairy cattle. Consequently, there is considerable interest to further research on this compound to provide data that could potential allow for it use to mitigate enteric CH_4 emissions from ruminant production.

1.6. Hypothesis and objectives

The overall hypothesis is that NOP would reduce CH_4 emissions from beef cattle consuming high forage (i.e., backgrounding) diets in short and long-term experiments because it is a structural analog of methyl-CoM and, therefore, could act as a competitive inhibitor of MCR. Ruminal CH_4 depression would not have a detrimental effect on ruminal fermentation because rumen microbes have metabolic pathways other than methanogenesis to redirect MH, such as propionate. Furthermore, it was hypothesized that use of NOP together with the ionophore MON would further decrease CH_4 production because of the different modes of action of these compounds.

The main objectives of this research were: 1) to evaluate the potential of NOP to reduce enteric CH_4 production of beef cattle; 2) determine effects of NOP on intake, digestibility, ruminal fermentation and microbial community; 3) determine whether reductions in CH_4 production using NOP are sustained over time; and 4) evaluate the effects of NOP in combination with MON on CH_4 and H_2 production, rumen fermentation, microbial protein synthesis and microbial community using the rumen simulation technique (Rusitec).

1.4.7. Literature cited

Abecia, L., Toral, P. G., Martín-García, A. I., Martínez, G., Tomkins, N. W. Molina-Alcaide, E., Newbold, C. J., and Yañez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95:2027–2036.

Alexandratos, N., and J. Bruinsma. 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working paper No. 12-03. Rome, FAO.

Animut, G., Puchala, R., Goetsch, A. L., Patra, A. K., Sahlu, T., Varel, V. H. and Wells, J., 2008. Methane emission by goats consuming diets with different levels of condensed tannins from Lespedeza. Anim. Feed Sci. Technol. 144:212–227.

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

Bannink, A., and Tamminga S. 2005. Rumen Function. In Quantitative aspects of ruminant digestion and metabolism, 2nd ed., pp. 263-268 [J. Dijkstra, J.M. Forbes, J. France, editors]. CABI Publishing. Cambridge, MA. USA.

Bauchop, T. 1967. Inhibition of rumen methanogenesis by methane analogues. J. Bacteriol. 94:171–175.

Balch, W. E., and Wolfe, R. S. 1979. Transport of coenzyme M (2-mercaptoethanesulfomc acid) in *Methanobacterium ruminantium*. J. Bacteriol. 137: 264–237.

Bayaru, E., Kanda, S., Kamada, T., Itabashi, H., Andoh, S., Nishida, T., Ishida, M., Itoh, T., Nagara, K., and Isobe, Y. 2000. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. Anim. Sci. J. 72:139-146.

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

Beauchemin, K. A., McGinn, S. M. Martinez, T. F., and T. A. McAllister. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85:1990–1996.

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

Belanche, A., de la Fuente G., and Newbold C. J. 2014. Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiol. Ecol. 90:663–677.

Cersosimo, L. M., and Wright A. D. G. 2015. Rumen methanogens. In Rumen microbiology: From evolution to revolution [A. K. Puniya, R. Singh, D. N. Kamra, editors]. Springer. New Delhi, India.

Chalupa, W. 1997. Manipulating rumen fermentation. J. Anim. Sci. 46:585–599.

Czerkawski, J. W., and Breckenridge, G. 1975a. New inhibitors of methane production by rumen micro-organisms. Development and testing of inhibitors *in vitro*. Br. J. Nutr. 34:429–446.

Czerkawski, J. W., and Breckenridge, G. 1975b. New inhibitors of methane production by rumen micro-organisms. Experiments with animals and other practical possibilities. Br. J. Nutr. 34:447–57.

DasSarma, S., J.A. Coker, and P. DasSarma. 2009. Archaea - Overview. In Desk Encyclopedia of Microbiology, 2nd edition, [M. Schaechter, editor] Academic Press p. 118-139.

Demeyer, 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut. In Rumen microbial metabolism and ruminant digestion [J. P. Jouany, editor]. INRA. Paris, France.

Duval, S., and Kindermann, M. 2012. Use of nitrooxy organic molecules in feed for reducing enteric methane emissions in ruminants, and/or to improve ruminant performance. World Intellectual Property Organization. International Patent Application WO 2012/084629 A1.

de Bok, F. A. M., Plugge, C. M., and Stams, A. J. M. 2004. Interspecies electron transfer in methanogenic propionate degrading consortia. Water Res. 38:1368–1375.

Dohme, F., Machmüller, A., Wasserfallen, A., and Kreuzer, M. 2001. Ruminal methanogenesis as infuenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32:47–51.

Environment Canada, 2015. National Inventory Report 1990–2013. Greenhouse gas sources and sinks in Canada, The Canadian Government's Submission to the UN Framework Convention on Climate Change, Part 1.

EPA. 2010. Methane and Nitrous Oxide Emissions from Natural Sources . U.S. Environmental Protection Agency, Washington, DC, USA.

EPA. 2012. Global anthropogenic non-CO_2 greenhouse gas emissions: 1990 – 2030. EPA 430-R-12-006. U.S. Environmental Protection Agency. Office of Atmospheric Programs, Climate Change Division. Washington, DC.

Ermler, U., Grabarse, W., Shima, S., Goubeaud, M., and Thauer, R. K. 1997. Crystal structure of methyl-coenzyme M reductase: the key enzyme of biological methane formation. Science. 278:1457–62.

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

France, J. and Dijkstra, J. 2005. Volatile fatty acid production. In Quantitative aspects of ruminant digestion and metabolism, 2nd ed., pp. 156-175 [J. Dijkstra, J.M. Forbes, J. France, editors]. CABI Publishing. Cambridge, MA. USA.

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

Goel, G., and Makkar, H. P. S. 2012. Methane mitigation from ruminants using tannins and saponins. Trop. Anim. Health. Prod. 44:729–739.

Golden, R. J., Holm, S. E., Robinson, D. E., Julkunen, P. H., and Reese, E. A., 1997. Chloroform mode of action: implications for cancer risk assessment. Reg. Toxicol. Pharm. 26:142–155.

Gunsalus, R. P., Romesser, J. A., and Wolfe, R. S. 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. 17:2374–2377.

Grainger, C., Auldist, M. J., Clarke, T., Beauchemin, K. A., McGinn, S. M., Hannah, M. C. Eckard, R. J., and Lowe, L. B. 2008. Use of monensin controlled-release capsules to reduce methane emissions and improve milk production of dairy cows offered pasture supplemented with grain. J. Dairy Sci. 91:1159–1165.

Grainger, C., Clarke, T., Auldist, M. J., Beauchemin, K. A., McGinn, S. M., Waghorn, G. C., and Eckard, R. J. 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce

methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 89:241–251.

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

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

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

Haisan, J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R., and Oba, M., 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97, 3110–3119.

Hegarty, R. S., Gerdes, R., 1999. Hydrogen production and transfer in the rumen. Rec. Adv. Anim. Nutr. Austr. 12:37–44.

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

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

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R. O., Kindermann, M., and Duval, S. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. U. S. A. 112:10663–10668.

Holtshausen, L., Chaves, A. V., Beauchemin, K. A., McGinn, S. M., McAllister, T. A., Odongo, N. E., Cheeke, P. R., and Benchaar, C. 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows, J. Dairy Sci. 92:2809–2821.

Hook, S. E., Wright, A. G., and McBride, B. W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea. 2010:1–11.

Hulshof, R. B. A., Berndt, A., Gerrits, W. J. J., Dijkstra, J., van Zijderveld, S. M., Newbold, J, R., and Perdok, H. B. 2015. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci. 90:2317–2323.

Immig, I., Demeyer, D., Fiedler, D., Van Nevel, C., and Mbanzamihigo, L. 1996. Attempts to induce reductive acetogenesis into a sheep rumen. Arch. Tierernaehr. 49:363–370.

IPCC, 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex, P. M. Midgley, editors]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp., doi:10.1017/CBO9781107415324.

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, L.A. Meyer, editors]. IPCC, Geneva, Switzerland, 151 pp.

Janssen, P. H., and Kirs, M. 2008. Structure of the archaeal community of the rumen. Appl. Environ. Microbiol. 74: 3619–3625.

Janssen, P. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160:1–22.

Jayanegara, A., Ridla, M., Laconi, E., and Nahrowi. 2014. Can plant saponins lower methane emissions without hampering the nutrient digestibility of ruminants. Proceedings of the 16th AAAP Animal Science Congress Vol. II. 10-14 November 2014, Gadjah Mada University, Yogyakarta, Indonesia.

Johnson, E. D., Wood, A. S., Stone, J. B., and Moran Jr., E. T. 1972. Some effects of methane inhibition in ruminants (steers). Can. J. Anim. Sci. 52:703–712.

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

Keltjens, J. T. and Vogels, G. D. 1996. Metabolic regulation in methanogenic Archaea during growth on hydrogen and CO_2. Environ. Monit. Assess. 42:19–37.

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

Knight, T., Ronimus, R. S., Dey, D., Tootill, C., Naylor, G., Evans, P., Molano, G., Smith, A., Tavendale, M., Pinares-Patiño, C. S., and Clark, H. 2011. Chloroform decreases rumen

methanogenesis and methanogen populations without altering rumen function in cattle. Anim. Feed Sci. Technol. 166–167:101–112.

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

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54:519–543.

Li, L., Davis, J., Nolan, J., and Hegarty, R. 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. Anim. Prod. Sci. 52:653–658.

Liu, Y., and Whitman, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125: 171–189.

Lopez, S., McIntosh, F. M., Wallace, R. J., and Newbold, C. J. 1999. Effect of adding acetogenic bacteria on methane production by mixed rumen microorganisms. Anim. Feed Sci. Technol. 78:1–9.

Lovett, D., Lovell, S., Stack, L., Callana, J., Finlay, M., Conolly, J., and O'Mara, F. P. 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.

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

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

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

McBride, B. C., and Wolfe, R. S. 1971. A new coenzyme of methyl transfer, coenzyme M. Biochem.10:2317–2324.

McCrabb, G. J., Berger, K. T., Magner, T., May, C., and Hunter, R. A.1997. Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. Aust. J. Agric. Res. 48:323–329.

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

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., and McSweeney, C. S. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108:482–491.

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

Nollet, L., Demeyer, D., and Verstraete, W. 1997. Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive

acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. Appl. Environm. Microbiol. 63:194–200.

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

Patra, A. K. 2012. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ. Monit. Assess. 184:1929–1952.

Patra, A. K. 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. Livest. Sci.155:244–254.

Plaa, G.L. 2000. Chlorinated methanes and liver injury: highlights of the past 50 years. Annu. Rev. Pharm. Toxicol. 40:43–65.

Prakash, D., (Doctoral dissertation) 2014. Methyl-coenzyme M reductase: elucidating the process of activation and study of the effect of themethanogenesis inhibitor 3-nitrooxypropanol. Auburn University, https://etd.auburn.edu/handle/10415/4463.

Puchala, R., Min, B. R., Goetsch, A. L., and Sahlu, T. 2005. The effect of a condensed tannin–containing forage on methane emission in goats, J. Anim. Sci. 83:182–186.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of incremental rumen doses of 3-nitrooxypropanol onmethane production, digestion and rumen parameters in lactating dairy cows. J. Dairy Sci. 97, 3777–3789.

Robinson, J. A., Strayer, R. F., and Tiedje, J. M. 1981. Method for measuring dissolved hydrogen in anaerobic ecosystems: Application to the rumen. Appl. Environ. Microbiol. 41:545–548.

Russell, J. B. 2002. Rumen microbiology and its role on ruminant nutrition. J. B. Russell, Ithaca, NY.

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

Saengkerdsub, S., and Ricke, S. 2014. Ecology and characteristics of methanogenic archaea in animals and humans. Crit. Rev. Microbiol. 40:97–116.

Santoso, B., Mwenya, B., Sar, C., Gamo, Y., Kobayashi, T., Morikawa, R., Kimura, K., Mizukoshi, H., Takahashi, J. 2004. Effects of supplementing galacto-oligosaccharides, *Yucca schidigera* or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livest. Prod. Sci. 91:209–217.

Sauer, F. D., Fellner, V., Kinsman, R. Kramer, J. K. G., Jackson, H. A., Lee, A. J., and Chen, S. 1998. Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. J. Anim. Sci. 76:906–914.

Sauvant, D., and Giger-Reverdin, S. 2009. Modélisation des interactions digestives et de la production de méthane chez les ruminants. INRA Prod. Anim. 22:375–384.

Sawyer, M. S., Hoover, W. H., and Sniffen, C. J. 1974. Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. J. Anim. Sci. 38:908–914.

Śliwiński, B. J., Soliva, C. R., Machmüller, A., Kreuzer, M. 2002. Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation. Anim. Feed Sci. Technol. 101:101–114.

Soliva, C. R., Amelchanka, S. L., Duval, S. M., Kreuzer, M., 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). Br. J. Nutr. 106:114–122.

St-Pierre, B., and Wright, A.-D. G. Diversity of gut methanogens in herbivorous animals. Animal 7:49–56.

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., and Sivakumaran, S. 2005. Methane production from *in vitro* rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis, Anim. Feed Sci. Technol. 123–124:403–419.

Taylor, C. D., and Wolfe, R. S. 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3$). J. Biol. Chem. 249:4879–4885.

Thauer, R. 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology 144:2377-2406.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W., and Hedderich R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nature Rev. Microbiol. 6:579–591.

Tomkins, N. W., Colegate, S. M., and Hunter, R. A. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49:1053–1058.

Trei, J. E., Parish, R. C., Singh, Y. K., and Scott, G. C. 1971. Effect of methane inhibitors on rumen metabolism and feedlot performance of sheep. J. Dairy Sci. 54:536 –539.

Ungerfeld, E. M., Rust, S. R., Boone, D. R., and Liu, Y. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. J. Appl. Microbiol. 97:520–526

Ungerfeld, E.M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6:1–17.

United Nations (UN). 2015. World Population Prospects: The 2015 Revision.

Van Nevel, C. J., Henderickx, H. K., Demeyer, D. I., and Martin, J. 1969. Effect of chloral hydrate on methane and propionic acid in the rumen. Appl. Microbiol. 17:695–700.

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

Vogels G. D., W. F. Hoppe, and C. K. Stumm. 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ Microbiol. 40:608-612.

Wang, C. J., Wang S. P., and Zhou, H. 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Anim. Feed Sci. Technol. 148:157–166.

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

Wood, J, M., Kennedy, F. S., and Wolfe, R. S. 1968. The reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. Biochem. 7:1707–1713.

Wood, T. A., Wallace, R. J. Rowe, A., Price, J., Yañez-Ruiz, D. R., Murray, P., and Newbold, C. J. 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Anim. Feed Sci. Technol. 152:62–71.

World Meteorological Organization (WMO). 2014. WMO Greenhouse Gas Bulletin: The State of Greenhouse Gases in the Atmosphere Based on Global Observations through 2013. 6 November 2014. No. 10. Available online at http://www.wmo.int/pages/prog/arep/gaw/ghg/GHGbulletin.html.

Zhou, Z., Meng, Q., and Yu, Z. 2011. Effects of methanogenic inhibitors on methane production and abundances of methanogens and cellulolytic bacteria in *in vitro* ruminal cultures. Appl. Environm. Microbiol. 77:2634–2639.

Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R., and Stahl, D. A. 2000. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. Environ. Microbiol. 2:632–643.

2. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle[1]

2.1. Introduction

Ruminants produce CH_4 as a byproduct of enteric fermentation (EPA, 2012) which represents a loss of energy to the animal that can vary from 2 to 12% of GE intake (Johnson and Johnson, 1995). Methane is an important GHG and livestock is responsible for 37% of global anthropogenic emissions (Steinfeld *et al*., 2006). Therefore, a reduction in CH_4 production is desirable from the standpoint of both animal productivity and environmental impact. Research groups around the world are working to develop compounds with a mode of action similar to BES, BCM, and chloroform, which are potent inhibitors of methanogenesis, but without toxic or harmful effects on the environment and effective in the long term (Hristov *et al*., 2013). Duval and Kindermann (2012) proposed the use of nitrooxy organic molecules in feed, such as NOP (Figure 2.1), to reduce enteric CH_4 emissions in ruminants. 3-Nitrooxypropanol is a structural analog to methyl-CoM, a cofactor involved in the terminal step of methanogenesis that transfers a methyl group to MCR (Shima and Thauer, 2005). Therefore, NOP is thought to inhibit MCR, similarly to BES (Van Nevel and Demeyer, 1995). 3-Nitrooxypropanol has been shown to reduce CH_4 production both *in vitro* (Romero-Perez *et al*., 2013) and *in vivo* when supplemented to sheep (Martinez-Fernandez *et al*., 2013) and dairy cattle (Haisan *et al*., 2013, 2014; Reynolds *et al*., 2014) with no signs of animal toxicity. However, NOP has not yet been evaluated in beef cattle. The objective of the present study was to evaluate the potential of NOP to reduce enteric

[1]A version of this chapter has been published. Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M., and Beauchemin, K. A., 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682–4693.

CH_4 production from beef cattle fed a backgrounding diet and determine the effects on digestibility, ruminal fermentation, and the ruminal microbial community. We hypothesized that supplementation with NOP would reduce CH_4 emissions without detrimental effects on ruminal fermentation and digestibility.

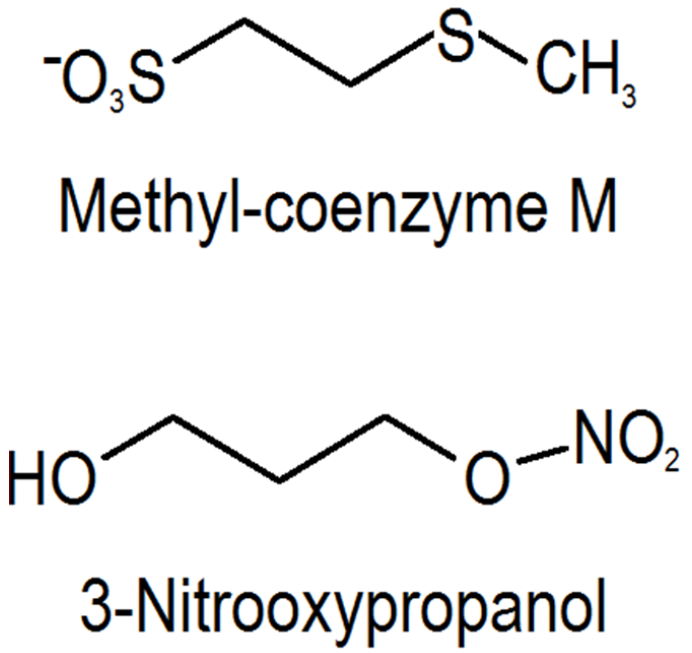

Figure 2.1. Structural formula of methyl-coenzyme M and 3-nitrooxypropanol. Adapted from Shima and Thauer (2005) and Duval and Kindermann (2012).

2.2. Materials and methods

The experiment was conducted at Agriculture and Agri-Food Canada's Research Centre in Lethbridge, AB, Canada. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Diet and animal management

Eight rumen cannulated Angus heifers (549 ± 64.3 kg [mean ± SD]) were used in this study. The basal diet (Table 2.1) consisted of 60% barley silage, 35% barley grain, and 5% vitamin–mineral supplement (DM basis). A backgrounding diet was used rather than a finishing diet because of the higher emissions associated with higher forage diets (Johnson and Johnson, 1995) and hence greater need for reduction. The diet was formulated to meet animal requirements for crude protein (CP), minerals, and vitamins (NRC, 1996). Additionally, each heifer received 600 g/d of a pellet containing 2.6 mg of melengesterol acetate (MGA; MGA-100 premix; Pfizer Animal Health, Pfizer Canada Inc., Kirkland, QC, Canada) top dressed onto the diet to suppress estrous activity. A total mixed ration (TMR) was prepared daily using a feed mixer (Data Ranger; American Calan Inc., Northwood, NH), and heifers were fed for ad libitum intake (at least 10% orts) once daily at 1300 h. When the animals were in the metabolic chambers and during digestibility measurements, the amount fed was restricted to 90% of *ad libitum* intake to avoid day-to-day variation in DMI within animal and to ensure the digestibility measurements were conducted at approximately similar intake as CH_4 emissions. From previous studies (McGinn *et al*., 2004; Beauchemin and McGinn, 2005), we anticipated a reduction in intake of the cattle when confined in metabolic chambers for measurement of CH_4 emissions.

Heifers were housed in a heated tie stall barn, fitted with rubber mattresses and bedded with wood shavings, except when CH_4 production (animals confined in a controlled environment facility) and digestibility measurements (tie stall barn with no bedding) took place. Heifers had access to an open dry lot for exercise daily.

Experimental design and dietary treatments

The experiment was designed as a replicated 4 × 4 Latin square with 2 groups, four 28-d periods, and 4 treatments. Heifers were grouped based on their pre-experimental BW. Lighter heifers were allocated to group 1 (497.4 ± 31.7 kg [mean ± SD]) and heavier heifers were allocated to group 2 (600.0 ± 40.6 kg [mean ± SD]). Dietary treatments were levels of NOP (DSM Nutritional Products, AG, Kaiseraugst, Switzerland) fed at 0, 0.75, 2.25, and 4.50 mg/kg of BW.

Heifers were adapted to their treatments gradually at the beginning of each period. Heifers receiving 4.5 mg NOP/kg of BW were supplemented with 0.75 mg NOP/kg of BW on d 1, 2.25 mg NOP/kg of BW on d 2, and the full amount from d 3. Heifers receiving 2.25 mg NOP/kg of BW received 0.75 mg NOP/kg of BW on d 1 and the full amount from d 2. Heifers supplemented with 0.75 mg NOP/kg of BW received the full amount starting d 1. The NOP was mixed with 13 g of a carrier containing 10 g ground barley, 2 g liquid molasses, and 1 g canola oil (as-fed basis), to improve palatability. The feed additive mixture was top dressed together with the MGA pellet onto the fresh total mixed ration daily at the feeding time. The NOP and carrier were consumed by animals within 10 min of presentation.

Table 2.1. Ingredient and chemical composition of the basal diet

Item	% of DM
Ingredient[1, 2]	
Barley silage[3]	60
Barley grain, dry-rolled	35
Barley grain, ground	2.688
Calcium carbonate	1.374
Canola meal	0.5
Salt	0.158
Urea	0.11
Molasses, dried	0.108
Feedlot vitamin-mineral premix[4]	0.055
Vitamin E (500,000 IU/kg)	0.004
Flavouring agent	0.003
Chemical composition[5]	
DM	46.7 ± 2.05
OM, % of DM	92.9 ± 0.43
CP, % of DM	11.7 ± 0.25
NDF, % of DM	37.6 ± 1.31
ADF, % of DM	20.6 ± .55
Starch, % of DM	31.8 ± 1.03
Fat, % of DM	2.7 ± 0.19

[1]All ingredients except barley silage and dry-rolled barley grain were provided as part of a pelleted supplement.

[2]Each heifer received 2.69 mg/d of melengesterol acetate as a pellet to suppress estrous activity. Pellet contained: MGA-100 premix (Pfizer Canada Inc., Kirkland, QC, Canada), 0.45%; ground barley grain, 95.99%; dried molasses, 2.51%; flavouring agent, 0.05% (DM basis). It was fed at 600 g/animal daily (as-is basis).

[3]Composition: DM, 34.5%; CP, 11.4%; NDF, 49.7%; ADF, 32.3%; starch, 16.1%; GE 5.6 Mcal/kg.

[4]Feedlot vitamin-mineral premix contained: $CaCO_3$, 35.01%; $CuSO_4$, 10.37%; $ZnSO_4$, 28.23%; EDDI 80, 0.15%; selenium 1% (10,000 mg Se/kg), 5.01%; $CoSO_4$, 0.1%; $MnSO_4$, 14.54%; vitamin A (500,000,000 IU/kg), 1.71%; vitamin D (500,000,000 IU/kg), 0.17% and vitamin E (500,000 IU/kg), 4.7%.

[5]Mean ± SD; n = 4.

Ruminal fermentation was monitored by collecting ruminal contents at 0 (prefeeding), 3, 6, 9, and 12 h after feeding on d 14. The heifers were moved to metabolic chambers to measure enteric CH_4 and CO_2 production for 3 consecutive days starting on d 18. Ruminal pH was continuously measured for 7 d from d 14 to 21, which coincided with the measurements of ruminal fermentation and enteric gas production. The heifers were returned to the heated tie-stall barn and apparent total tract digestibility was measured for 4 d from d 24 to 28. Based on information from a previous *in vitro* experiment done in our lab (Romero-Perez, Okine, McGinn, Guan, Oba, Duval, and Beauchemin, personal communication), we expected any carryover effects of NOP would dissipate within 5 d.

Sampling

Feed offered and refused were recorded daily for individual heifers. Dry matter intake was calculated using the DM contents of the basal diet and refusal samples.

Refusals were sampled from Monday to Friday, composited by week for each animal, and stored at –20°C until analyzed for DM and chemical composition. Sampling of the basal diet and barley silage was done 3 times per week to monitor DM content. Dried samples of the basal diet from week 4 (digestibility measurements) were pooled and stored for analysis. Dried samples for barley silage were pooled by period and stored until analysis. Dry ingredients including barley grain, vitamin–mineral supplement, and MGA pellet were sampled weekly. A subsample was used to determine DM content. Another subsample was composited by period and stored at –20°C until chemical analysis. The proportion of the ingredients in the basal diet (as-fed basis) were adjusted when the DM content of an ingredient varied by >3%.

Body weight

Body weights (not fasted) were measured at the beginning of the experiment, before and after the animals went into the metabolic chambers, and at the end of each period. The average BW for the before and after chamber weights was used when expressing enteric CH_4 production on the basis of BW. Body weights that were obtained at the beginning of the experiment were used for calculating the amount of NOP needed for Period 1. Thereafter, BW obtained before the chamber measurements were used to calculate the amount of NOP needed for the following period.

Enteric gas production

On d 18 of each period, heifers were moved to metabolic chambers (1 heifer/chamber) and production of CH_4 and CO_2 was measured during 3 consecutive days. The 2 groups of heifers were staggered by 1 week to allow gas measurements. Heifers were conditioned to the chambers before the beginning of the experiment to minimize stress. Each chamber measured 4.4 m wide by 3.7 m long by 3.9 m high (63.5 m3; model C1330; Conviron Inc., Winnipeg, MB, Canada). Within each chamber, the animal was housed in a stall equipped with a feeder and drinking bowl and fitted with a rubber mattress. Concentrations of CH_4 and CO_2 in the intake and exhaust air ducts were monitored using a CH_4 analyzer (model Ultramat 5E; Siemens Inc., Karlsruhe, Germany) and a CO_2/H_2O analyzer (model LI-7000; LI-COR Environmental, Lincoln, NE), respectively. Gas concentrations in the intake and exhaust air ducts of the chambers were monitored sequentially (3 or 4 min from the intake or from the exhaust ducts) for a total of 7 min/chamber, except for 1 chamber that was sampled for 6 min (3 min from the intake and 3 min from the exhaust duct). All chambers were sampled within 27 min, with an additional 3 min used to measure a zero reference gas (pure nitrogen gas). The gas sampling procedure was repeated every 30 min. Only the last minute of the 3 or 4-min sampling was retained for analysis to avoid any possible carryover contamination. The air flow in the intake and exhaust ducts was continually monitored using a duct mounted airflow measurement station (FE-1500-FX-12; Paragon Controls Inc., Santa Rosa, CA). The difference between the incoming and outgoing mass of CH_4 or CO_2 was used to calculate the amount generated in each chamber by each animal.

Each chamber door was opened daily for about 5 min while the gas concentrations were not being monitored. The chambers were opened sequentially to allow feeding and cleaning.

These interruptions had no effect on daily emissions because there was an 18- or 19-min reequilibration period before sampling resumed in which 4.2 turnovers of the volume of air in each chamber occurred. To account for between-chamber differences, each chamber was calibrated before and after the experiment by releasing known amounts of CH_4 and CO_2 and calculating recoveries. Calibration factors were then generated to correct emissions for each gas. Details of the chamber design and the calculation of CH_4 and CO_2 emissions were reported by McGinn et al. (2004).

Ruminal Variables

Starting on d 14, ruminal pH was measured every minute for 7 d using the Lethbridge Research Centre Ruminal pH Measurement System (LRCpH; Dascor, Escondido, CA). The system was standardized using buffers pH 4 and 7 at the start and end of each measurement period. The shift in millivolt reading from the electrodes between the start and end standardizations was assumed to be linear and was used to convert millivolt readings to pH units as described by Penner *et al*. (2006). Five milliliters of filtered ruminal fluid was added to 1 mL of 1% sulfuric acid (vol/vol) for NH_3 determination. Another 5 mL of the filtered ruminal fluid was added to 1 mL of 25% metaphosphoric acid (wt/vol) for VFA determination. These samples were stored at –20°C until analyzed. Whole rumen contents were also collected at 0 (prefeeding), 6, and 12 h after feeding, immediately frozen using liquid nitrogen, and stored at –80°C until analyzed for protozoa, methanogens, and bacteria.

Total collection for digestibility

Apparent total tract digestibility was estimated by total collection of feces. Heifers were fitted with urinary catheters (Bardex Lubricath Foley catheter, 75 cm3 and 12 mm; Bard Canada Inc., Oakville, ON, Canada) to ensure separation of urine and feces. Samples (approximately 10% of total) of the daily feces were dried for 72 h at 55°C in a forced air oven to a constant weight and composited by pooling the dried daily feces by animal within period based on their respective DM contents. The composited fecal samples were analyzed for contents of ash, nitrogen, NDF, ADF, starch, and GE.

Laboratory analyses

Analyses were performed on each sample in duplicate; when the coefficient of variation was >5%, the analysis was repeated. The DM content was determined by drying for 72 h at 55°C. The dried samples (feeds, refusals, and feces) were ground in a Wiley mill (A.H. Thomas, Philadelphia, PA) through a 1-mm screen. Analytical DM was determined by drying at 135°C for 2 h (AOAC, 2005; method 930.15) followed by hot weighing. The OM was calculated as the difference between DM and ash (AOAC, 2005; method 942.05). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined according to Van Soest *et al*. (1991) with heat-stable amylase and sodium sulfite used in the NDF procedure. Crude fat (AOAC, 2006; method 2003.05) was determined using ether extraction (Extraction Unit E-816 HE; BŰCHI Labortechnik AG, Flawil, Switzerland). The GE content was determined using a bomb calorimeter (model E2k; CAL2k, Johannesburg, South Africa). The 1-mm ground samples were reground using a ball grinder (Mixer Mill MM2000; Retsch, Haan, Germany) before determination of nitrogen and starch contents. The nitrogen content was determined by flash

combustion with gas chromatography and thermal conductivity detection (Carlo Erba Instrumentals, Milan, Italy). Starch content was determined by enzymatic hydrolysis of α-linked glucose polymers as described by Chung *et al*. (2011). Ruminal VFA was quantified using GLC (model 5890; Hewlett-Packard, Wilmington, DE) with a capillary column (30 m by 0.32 mm by 1 μm; ZB-FFAP; Phenomenex Inc., Torrance, CA) and flame ionization detection. Crotonic acid was used as internal standard. The oven temperature was maintained at 150°C for 1 min, increased by 5°C/min to 195°C, and held at this temperature for 2.5 min. The injector temperature was 225°C, the detector temperature was 250°C with helium as the carrier gas. Ruminal NH_3 concentration was determined by the salicylate–nitroprusside–hypochlorite method using segmented flow analyzer (Rhine *et al*., 1998).

Frozen rumen contents (1 g) were thawed on ice and processed fordeoxiribunocleic acid (DNA) extraction. The bead-beating method was used to extract total DNA from the rumen digesta using the protocol outlined by Guan *et al*. (2008). After extraction, the concentration and quality of DNA were measured at 260 and 280 nm by using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Total bacteria and methanogens in the rumen digesta were estimated by measuring the copy number of 16S ribosomal ribonucleic acid (rRNA) genes using quantitative PCR (qPCR). The primer pair U2 (forward, 5′-ACTCCTACGGGAGGCAG-3′, and reverse, 5′-GACTACCAGGGTATCTAATCC-3′; Stevenson and Weimer, 2007) was used to detect the total copy number of bacterial 16S rRNA genes, and the primer pair uniMet (forward, 5′-CCGGAGATGGAACCTGAGAC-3′, and reverse, 5′-CGGTCTTGCCCAGCTCTTATTC-3′; Zhou *et al*., 2009) was used to detect the total copy number of methanogenic 16S rRNA genes. The qPCR was performed with SYBR Green chemistry (Fast SYBR Green master mix; Applied

Biosystems, Foster City, CA), using the StepOnePlus real-time PCR system (Applied Biosystems). The amplification program included a fast cycle and a melting curve section. For total bacteria, the program consisted of 95°C for 5 min followed by 40 cycles at 95°C for 20 s and 62°C for 30 s. The program for total methanogens included 95°C for 20 s for initial denaturation and then 40 cycles of 95°C for 3 s followed by annealing/extension for 30 s at 60°C. The final melting curve detection of both microbes were the same, with 95°C for 15 s, 60°C for 1 min, and then an increase to 95°C with fluorescence collection at 0.1°C intervals. The standard curves were constructed using a serial dilution of plasmid DNA from clones containing the full length 16S ribosomal DNA insert of *Butyrivibrio hungatei* (Chen *et al*., 2011) and *Methanobrevibacter* sp. strain AbM4 (Zhou *et al*., 2009), respectively. The final copy number of 16S rRNA genes of targeted microbes per gram of rumen contents was calculated based on the formula according to Chen *et al*. (2012).

The total protozoa were estimated by analyzing the total copy number of 18S rRNA genes using primer pair P-SSU-316F (5′-GCTTTCGWTGGTAGTGTATT-3′) and P-SSU-539R (5′-CTTGCCCTCYAATCGTWCT-3′; Sylvester *et al*., 2004) and using qPCR with SYBR Green chemistry. The standard curve was constructed using plasmid DNA containing a cloned sequence (223 bp) amplified by the same primer set, which was confirmed by basic local assignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The initial copy number for the standard curve were calculated based on the formula $(NL \times A \times 10^{-9})/(660 \times n)$, in which NL is the Avogadro constant (6.02×10^{23} molecules per mol), A is the molecular weight of the molecule in standard, and n is the length of the amplicon (bp). The serial dilutions were assigned from 10^{-3} to 10^{-8}. The qPCR was performed using a fast cycle and a melting curve section. The program was 95°C for 20 s for initial denaturation and then 40 cycles of 95°C for 3 s followed by

annealing/extension for 30 s at 60°C. The final melting curve detection of protozoa was 95°C for 15 s, 60°C for 1 min, and then an increase to 95°C with fluorescence collection at 0.1°C intervals. Similarly, the final copy number of 18S rRNA genes of targeted microbes per gram of rumen content was obtained using the same method as described above.

Calculations and statistical analysis

Heifer was the experimental unit for all variables. The daily CH_4 flux was determined for each heifer and expressed as a portion of GE and DE intake on the same day, assuming that energy content of CH_4 is 13.3 Mcal/kg. Data for ruminal pH were summarized by day for minimum, maximum, and average pH. Because protozoa, methanogens, and bacteria data were not normally distributed, a $\log_{10}$ transformation was applied before analysis with the inverse $\log_{10}$ least square mean reported. Data were analyzed using a mixed model procedure (SAS Inst. Inc., Cary, NC) that included the fixed effect of the treatment (0, 0.75, 2.25, or 4.5 mg NOP/kg of BW), sampling time (hour or day), and their interactions. Group, period within group, and heifer within group were considered random effects. Time of sampling was considered a repeated effect in the model with heifer × group × period × treatment as the subject. Methane was expressed on the basis of DMI, GE intake, and DE intake.

The variance components were estimated using the REML method and degrees of freedom were adjusted using the Kenward-Roger option. The PDIFF option adjusted by the Tukey method was used to separate means. The covariance structure was selected based on the lowest Akaike and Bayesian information criteria values. Because NOP levels among treatments were not equally spaced, linear, quadratic, and cubic effects of treatments were tested using

orthogonal contrasts. Treatment differences and trends were declared significant at $P \leq 0.05$ and $P \leq 0.10$, respectively.

2.3. Results

Cubic effects were not statistically significant for any of the variables measured, and therefore only linear and quadratic effects are reported. There were no interactions between the effects of sampling time and NOP levels; therefore, only means for the main effect of treatment are presented.

Increasing levels of NOP decreased *ad libitum* DMI in a linear ($P = 0.02$) and quadratic ($P = 0.07$) manner (Table 2.2). However, reduction in DMI only differed from the control ($P = 0.03$) when 2.25 mg NOP/kg BW was supplemented. Despite the reduction in *ad libitum* DMI, BW and average daily gain (ADG) were not affected by level of NOP ($P \geq 0.21$). The BW for heifers consuming 0, 0.75, 2.25, and 4.5 mg NOP/kg was 619, 618, 616, and 621 kg, respectively, while ADG was 1.03, 0.96, 0.87, and 0.87 kg/d, respectively.

Adding NOP to the diet tended ($P = 0.10$) to affect DM digestibility in a quadratic manner ($P = 0.05$), with slightly lower DM digestibility for 0.75 and 2.25 mg NOP/kg BW and slightly greater DM digestibility for 4.5 mg NOP/kg BW compared with the control (Table 2.2). Consequently, there was a trend for a quadratic ($P = 0.06$) response in OM digestibility and a trend for a linear ($P = 0.08$) and quadratic ($P = 0.07$) response in GE digestibility to NOP. However, NOP supplementation had no effect ($P \geq 0.14$) on OM, CP, NDF, ADF, and starch digestibility.

Minimum ruminal pH linearly increased with increasing levels of NOP ($P = 0.02$) but only differed from control when 2.25 mg NOP/kg of BW was supplemented (Table 2.3). As a

result, the pH range was linearly decreased ($P = 0.04$) with NOP supplementation. Mean and maximum pH remained unchanged with NOP inclusion. The diurnal ruminal pH pattern (Figure 2.2) for treatments showed consistent reduction of pH after feeding reaching nadir 7 to 9 h later.

Subsequently, pH started to increase with a slight drop 19 h after feeding. Ruminal pH was consistently greater than control when 2.25 and 4.5 mg NOP/kg of BW were supplemented. Total VFA linearly decreased ($P = 0.04$) when NOP was increased. Molar proportion of acetate decreased ($P < 0.01$) and that for propionate increased ($P < 0.01$) with increasing levels of NOP. Compared with the control, acetate proportion was decreased by 9 and 15% with 2.25 and 4.5 mg NOP/kg of BW, respectively, and propionate proportion was increased by 22% with the inclusion of 4.5 mg NOP/kg of BW. This change caused a reduction in the acetate to propionate ratio, which was decreased by 3, 17, and 38% when NOP was supplemented at 0.75, 2.25, 4.5 mg/kg of BW, respectively. The molar proportion of butyrate, valerate, and isovalerate linearly increased ($P \leq 0.04$) and isobutyrate tended to linearly increase ($P = 0.09$) with increasing levels of NOP. The NH_3 concentration was unchanged ($P = 0.34$) when NOP was included.

Total copy numbers of protozoa, methanogens, and bacteria were not affected by inclusion level of NOP ($P \geq 0.31$; Table 2.4). Time had a significant effect on total copy number of bacteria and methanogens ($P < 0.03$); however, the interaction between NOP and time was not significant for any of the microorganisms studied ($P \geq 0.38$).

No effect on DMI was observed when animals were in the chambers ($P = 0.21$; Table 2.5). However, DMI was 27% lower in the chambers than when animals were fed *ad libitum* (Table 2.2) due to the decreased energy expenditure and stress associated with isolation in the chambers.

Table 2.2. *Ad libitum* DMI and nutrient digestibility of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

	Treatment[1]					*P*-value[2]		
Item	0	0.75	2.25	4.5	SEM	Trt	Lin	Quad
Ad libitum DMI, kg/d[3]	12.0[a]	11.7[ab]	11.3[b]	11.4[ab]	0.9	0.03	0.02	0.07
Digestibility, %								
DM	68.4	67.5	67.3	69.7	1.07	0.10	0.13	0.05
OM	70.8	70.1	69.7	71.9	1.02	0.14	0.17	0.06
CP	63.8	62.9	62.8	66.9	1.69	0.22	0.11	0.18
NDF	53.8	52.2	53.1	54.8	1.83	0.36	0.25	0.26
ADF	44.7	42.4	43.6	45.2	1.79	0.41	0.42	0.26
Starch	94.2	94.9	94.1	94.2	0.87	0.89	0.76	0.95
GE	66.4	65.6	65.4	68.2	1.19	0.10	0.08	0.07

[1]Expressed as mg of 3-nitrooxypropanol per kg of animal BW.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[3]*Ad libitum* intake from d 1 to 16.

[a, b, c] Within a row, means without a common superscript differ ($P < 0.05$).

Table 2.3. Rumen fermentation of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

Variable	Treatment[1]					*P*-value[2]		
	0	0.75	2.25	4.5	SEM	Trt	Lin	Quad
Ruminal pH								
Minimum	5.83^{b}	6.00^{ab}	6.04^{a}	6.06^{a}	0.10	0.04	0.02	0.14
Mean	6.46	6.54	6.57	6.55	0.05	0.28	0.19	0.17
Maximum	6.96	6.98	6.97	6.91	0.06	0.70	0.35	0.52
Range[3]	1.13	0.99	0.93	0.85	0.09	0.08	0.02	0.38
Total VFA, m*M*	160.5	159.1	148.4	147.7	9.13	0.16	0.04	0.41
VFA, mol/100 mol								
Acetate (A)	61.8^{a}	60.8^{a}	56.3^{b}	52.6^{c}	0.99	<0.01	<0.01	0.57
Propionate (P)	19.3^{b}	19.4^{b}	21.4^{b}	26.1^{a}	1.16	<0.01	<0.01	0.23
Butyrate	12.3^{b}	12.7^{b}	15.2^{a}	13.8^{ab}	0.60	0.003	0.01	0.004
Valerate	1.74^{b}	1.83^{ab}	1.85^{ab}	2.10^{a}	0.09	0.03	0.004	0.52
Isobutyrate	1.01	1.01	1.08	1.06	0.04	0.15	0.09	0.17
Isovalerate	1.95^{b}	2.27^{ab}	2.256^{ab}	2.30^{a}	0.15	0.04	0.04	0.13
A:P ratio	3.33^{a}	3.23^{ab}	2.74^{b}	2.08^{c}	0.21	<0.01	<0.01	0.76
NH_3, m*M*	5.62	5.67	5.76	5.05	0.56	0.68	0.34	0.46

[1]Expressed as mg of 3-nitrooxypropanol per kg of animal BW.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[3]Range = maximum ruminal pH − minimum ruminal pH.

[a, b, c] Within a row, means without a common superscript differ ($P < 0.05$).

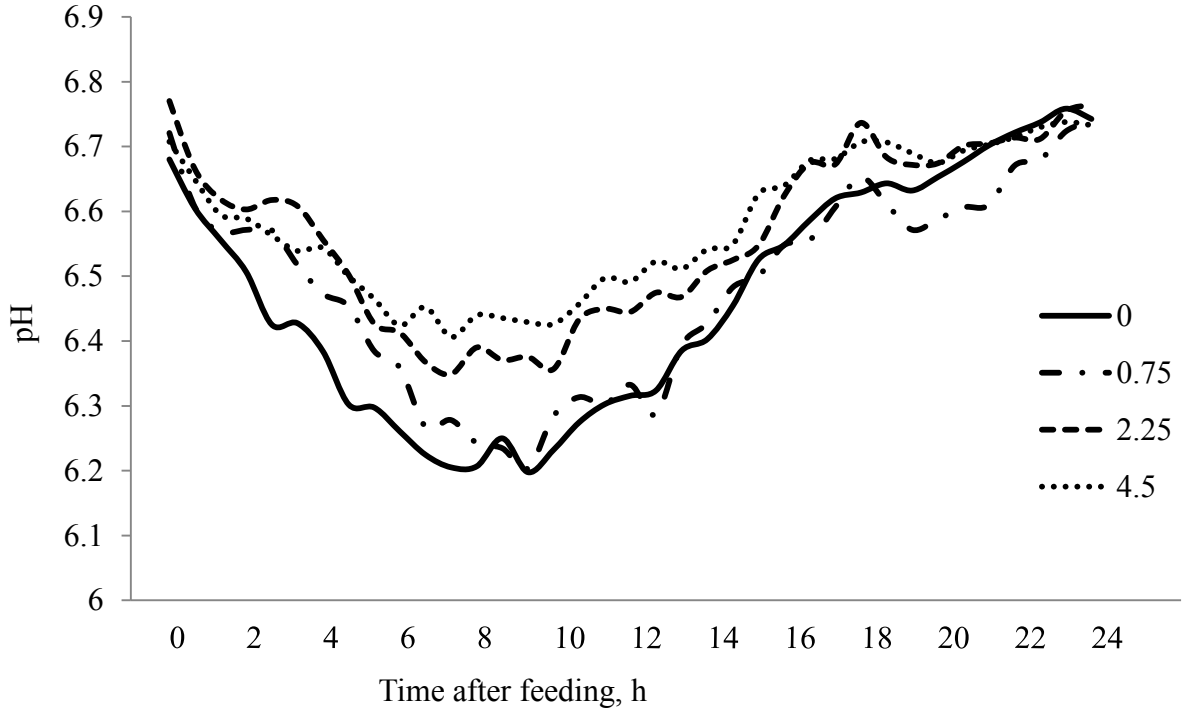

Figure 2.2. Daily pattern of rumen pH from beef cattle fed a backgrounding diet added with 0, 0.75, 2.25, and 4.5 mg of 3-nitrooxypropanol per kg of animal BW.

Regardless of the manner in which CH_4 emissions were expressed, a reduction was observed when 4.5 mg NOP/kg of BW was included in the diet (Table 2.5). Total CH_4 emissions per animal (g/d) were linearly ($P < 0.01$) decreased by 3, 13, and 38% with the inclusion of 0.75, 2.25, and 4.5 mg NOP/kg of BW, respectively, compared with the control. The CH_4 production expressed on the basis of DMI or as a percentage of GE or digestible energy (DE) intake was significantly decreased by 33, 33, and 35%, respectively, when the greatest level (4.5 mg NOP/kg of BW) of NOP was included ($P < 0.01$). Total CO_2 production was not affected by NOP ($P = 0.82$), however; CO_2 production 2 h after feeding was linearly increased with increasing level of NOP.

Table 2.4. Rumen protozoa, bacteria and methanogens of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

Variable	Treatment[1]					*P*-value[2]		
	0	0.75	2.25	4.5	SEM	Trt	Time	Trt × Time
Protozoa, × 10^5 copies/g	3.98	5.56	4.11	5.39	1.11	0.94	0.17	0.97
0 h	4.94	6.14	4.14	6.67	1.71			
6 h	3.73	5.75	4.63	6.17	1.71			
12 h	3.28	4.8	3.57	3.35	1.71			
Bacteria, × 10^{10} copies/g	1.34	1.25	1.98	1.26	0.57	0.55	<0.01	0.38
0 h	2.16	1.88	3.14	1.35	0.54			
6 h	0.84	0.85	1.52	0.57	0.54			
12 h	1.02	1.03	1.27	1.86	0.54			
Methanogens × 10^8 copies/g	6.2	6.92	7.42	3.66	1.58	0.31	0.03	0.42
0 h	7.74	6.52	7.46	4.24	1.21			
6 h	4.07	6.56	7.6	2.37	1.21			
12 h	6.81	7.65	7.2	4.39	1.21			

[1]Expressed as mg of 3-nitrooxypropanol per kg of animal BW.

[2]Trt = treatment effect.

Table 2.5. Enteric methane and carbon dioxide emissions of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

	Treatment					P-value[1]		
Item	0	0.75	2.25	4.5	SEM	Trt	Lin	Quad
DMI, kg/d[2]	8.5	8.7	8.6	8.1	0.63	0.52	0.21	0.46
CH_4 intensity								
CH_4, g/ d	206.8[a]	199.2[ab]	180.2[b]	129.1[c]	22.9	<0.01	<0.01	0.25
CH_4, g/kg DMI	24.62[a]	23.54[a]	22.33[a]	16.48[b]	1.80	<0.01	<0.01	0.21
CH_4,[3] % of GE intake	6.49[a]	6.20[a]	5.89[a]	4.34[b]	0.47	<0.01	<0.01	0.20
CH_4,[4]% of DE intake	9.77[a]	9.32[a]	9.02[a]	6.34[b]	0.69	<0.01	<0.01	0.12
CO_2, kg/d	7.37	7.24	7.41	7.35	0.55	0.80	0.82	0.90
CO_2 2 h post-feeding, kg	0.57[b]	0.60[ab]	0.61[ab]	0.70[a]	0.67	<0.01	<0.01	0.59

[1]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[2]Intake during CH_4 measurements.

[3]GE intake calculated from DMI in the chambers and GE content of the TMR.

[4]DE intake calculated from DMI in the chambers and digestibility of GE measured during the digestibility phase.

[a, b, c] Within a row, means without a common superscript differ ($P < 0.05$).

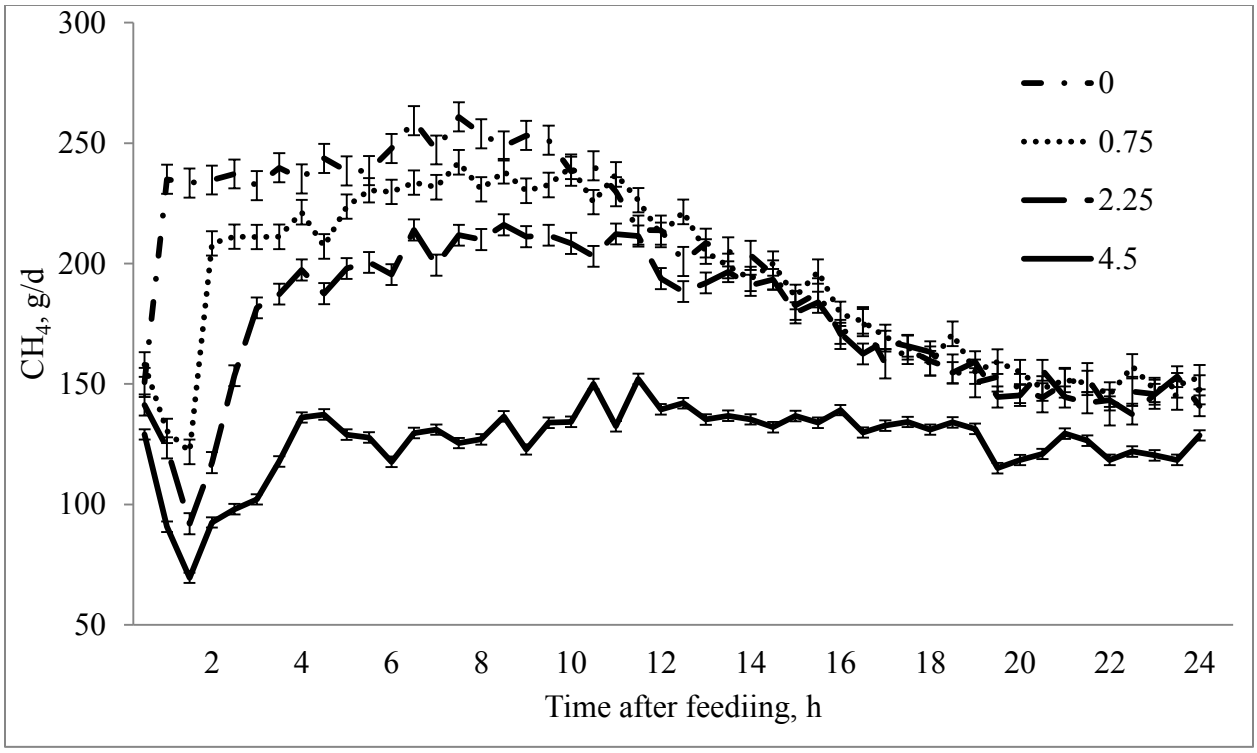

Figure 2.3. Daily pattern of methane production from beef cattle fed a backgrounding diet with 0, 0.75, 2.25, and 4.5 mg of 3-nitrooxypropanol per kg of animal bodyweight. Error bars indicate SEM.

In addition to examining the total enteric CH_4 production, the diurnal CH_4 production pattern was examined (Figure 2.3). For control animals, enteric CH_4 production rapidly increased 1 h after feeding. It continued to gradually increase reaching peak production 6 to 8 h after feeding and then it declined over the rest of the day. A negative transitory effect on CH_4 production was evident for a period of 2 h after feeding the different levels of NOP. Thereafter, CH_4 production followed a similar postprandial CH_4 production pattern as the control, such that 13 h after feeding, CH_4 production for animals receiving 0.75 and 2.25 mg NOP/kg of BW was similar to that for control. This was not the case for the highest level of supplementation (4.5 mg

NOP/kg BW), for which CH_4 production remained consistently lower at all times compared to other treatments.

2.4. Discussion

This study demonstrates that enteric CH_4 production by beef cattle fed a backgrounding diet was decreased when NOP was supplemented to the diet. The reduction in CH_4 production is consistent with the development of NOP as an inhibitor of methanogenesis due to inhibition of MCR. Information about the use of NOP is limited; however, the literature indicates significant reductions of enteric CH_4 emissions when supplemented to ruminants. The first experiment to evaluate the effectiveness of NOP in food producing animals was done by Martinez-Fernandez *et al*. (2013). They supplemented 100 mg of NOP per day into the rumen of sheep consuming a diet consisting of alfalfa and oats in a 60:40 forage to concentrate ratio. On d 14, NOP significantly decreased CH_4 emissions by 24.7% and 2 week later, on d 30, there was still a tendency for reduced emissions. Haisan *et al*. (2014), using lactating Holstein cows, observed a substantial reduction of 60% in CH_4 production when 2.5 g/d of NOP was supplemented to dairy cows consuming a 38% forage diet (DM basis). In another study, a reduction of 41.5% was observed when the same dose was supplemented to dairy cows consuming a 60% forage diet (Haisan *et al*., 2013). In both dairy studies, NOP was mixed into the ration by hand. The reduction in CH_4 production (33%) obtained with the highest dose in our study was lower when compared to the reduction reported by Haisan *et al*. (2014), even though the dose used in our study was similar (4.5 mg NOP/kg of BW equivalent to 2.7 g/heifer) and DMI was lower (8.1 vs. 19.3 kg/d) than in their study. The dose of NOP supplemented per kilogram of feed was 2.5 times higher for our beef experiment and suggests that factors such as the method that NOP is administered to

animals (e.g., mixed with the feed or top dressed) as well as animal type (e.g., beef, dairy, sheep) can affect the CH_4 response.

In a study conducted by Reynolds *et al*. (2014) using dairy cows fed a 43% forage diet (DM basis) and administered 0.5 or 2.5 g NOP/animal directly into the rumen, a significant reduction of 4.5 and 6.7%, respectively, in CH_4 production corrected for DMI was observed. The 4.5% reduction obtained when supplementing 0.5 g NOP/animal was similar to the reduction observed in our study (4.3%) with the lowest NOP dose (0.75 mg NOP/kg of BW equivalent to 0.45 g/animal). However, in our study the reduction at the low supplementation level was not significant when compared with the control.

The marked reduction in CH_4 emissions observed 2 h after feeding in our study was similar to that observed by Reynolds *et al*. (2014) with 2.5 g NOP/d supplemented to dairy cows. They observed a pronounced reduction (2–3 h) in CH_4 concentration in exhaust air (inferring decreased emissions) when dosing NOP before the afternoon feeding and a delay in the increase of CH_4 production after the morning feeding as compared to the control treatment. Although the reductions in CH_4 were pronounced in that study, their transitory nature suggests there is need of a continuous infusion of NOP into the rumen when supplemented to dairy cows. The authors in that study hypothesized that the compound may be absorbed, metabolized, or washed out of the rumen. The washout hypothesis from the rumen is very likely; however, washout would be expected to be more pronounced in dairy than in beef cattle. Based on the higher DMI observed in dairy experiments where NOP was supplemented, a higher dilution rate is expected compared to beef cattle. Dilution rate of the liquid phase is positively affected by DMI (Seo *et al*., 2006) wherein NOP is expected to be present.

The more pronounced effect of NOP on reducing CH_4 production during the first 2 h after feeding and the rapid increase in CH_4 after this time is in agreement with the suggestion that NOP is absorbed, metabolized, and/or washed out from the rumen. Therefore, continuous supplementation, rather than the pulse dose approach used in this study, may help prolong the effect of this compound on CH_4 reduction. Thoroughly mixing NOP with the diet, instead of top dressing the compound, could improve the potential benefits of feeding NOP by synchronizing the availability of the compound in the rumen with ruminal fermentation.

The percentage of GE intake lost as CH_4 for the control diet was similar to the value of 6.5% used by the International Panel on Climate Change Tier 2 methodology (IPCC, 2006). Beauchemin and McGinn (2005) reported that loses of CH_4 with backgrounding diets were higher than for finishing diets (7.4 vs. 3.4% of GE intake, respectively). In our study, cattle supplemented with the highest level of NOP had CH_4 emissions, expressed as kg CH_4/kg GE intake, typical of levels observed for finishing diets.

The daily CO_2 emissions in our study are greater than previous values reported by our group. McGinn *et al*. (2004) reported an average daily production of 3.44 kg of CO_2 per animal whereas Beauchemin and McGinn (2005) reported an average of 3.86 kg of CO_2 per animal. However, the animals used in those studies consumed less DM and BW was lower compared with the observation in our study. McGinn *et al*. (2004) observed a greater CO_2 production for beef cattle during the finishing phase compared to animals in the backgrounding phase and attributed this to greater energy intake and metabolic rate of the heavier cattle. The linear increase of CO_2 production 2 h after feeding with increasing levels of NOP is in agreement with the linear reduction observed for CH_4 emissions. Carbon dioxide and H_2 are substrates for methanogenesis. A decrease in CH_4 formation could cause an increase in CO_2 and H_2 if alternate

metabolic pathways are limited to use these gases as substrates. This has been previously demonstrated for H_2 when goats were supplemented with BCM (Mitsumori *et al*., 2012).

When NOP was administered to dairy cattle by Haisan *et al*. (2013) at 2.5 g per cow per day, no effect was observed on DMI or milk production; however, BW gain was increased. In a subsequent study by the same research team (Haisan *et al*., 2014), feeding 1.25 or 2.5 g NOP per cow did not affect DMI, milk production, or BW. Results from Martinez-Fernandez *et al*. (2013) showed no reduction in DMI when NOP was supplemented. Therefore, the decline in feed intake observed in our study is inconsistent with observations in dairy cows and sheep. 3-Nitrooxypropanol needs to be evaluated not only in metabolism studies, such as the present experiment, but also in production studies, to better assess its effects on the energy balance of ruminants.

The reduction in CH_4 production with NOP supplementation did not correspond to changes in diet digestibility. The reduction in *ad libitum* DMI when NOP was supplemented may indicate a potential palatability issue with NOP or could have resulted from negative feedback from the end products of digestion in the rumen. The linear reduction of total VFA concentration, acetate proportion, and acetate to propionate ratio, together with a linear increase in propionate, butyrate, valerate, isobutyrate, and isovalerate proportion with increasing levels of NOP, indicates changes in ruminal fermentation as a result of NOP supplementation. As observed in our study, antimethanogenic compounds reduce the acetate to propionate molar ratio in the rumen (Abecia *et al*., 2012). Similarly, a shift in fermentation pattern was observed in previous studies that evaluated NOP (Haisan *et al*., 2013, 2014; Martinez-Fernandez *et al*., 2013). A decrease in proportion of acetate acetate proportion with an increase in propionate and a decrease in acetate to propionate ratio has been reported with no changes in total VFA. Based

on the hypothesis that NOP would act as a competitive inhibitor of the enzyme MCR, thus inhibiting the reduction of methyl-CoM to CH_4 plus a coM-coB complex, a reduction in CH_4 production was expected together with the enhancement of an alternative metabolic pathway to utilize H_2. Considering that propionate is the principal alternative H_2 sink after CH_4 (McAllister and Newbold, 2008), a redirection of H_2 to propionate production was expected.

There was an interesting increase in minimum ruminal pH that needs further verification. Typically, CH_4 production and ruminal pH are positively associated across diets (Janssen, 2010), although within an animal, pH and CH_4 production are inversely related because pH drops and CH_4 production increases after feed consumption. In some studies, an increase in ruminal pH was observed (Nagaraja *et al*., 1981; Burrin and Britton, 1986; Melendez *et al*., 2004) when using monensin, an ionophore reported to reduce CH_4 emissions (McGinn *et al*., 2004; Odongo *et al*., 2007). Burrin and Britton (1986) related the increase in pH to a reduction in total VFA concentration. Recently, Reynolds *et al*. (2014) observed an increase in minimum ruminal pH together with a reduction in CH_4 and total VFA when 2.5 g of NOP were supplemented to dairy cattle. The linear reduction in total VFA concentration in the rumen observed in the present study could partially explain the increase in minimum ruminal pH when NOP was supplemented.

The lack of effect of NOP on the numbers of protozoa, bacteria, and methanogens is inconsistent with the observed reduction in CH_4 emissions. Haisan *et al*. (2013) and Martinez-Fernandez *et al*. (2013) did not observe changes in total bacteria and/or methanogens, even though a numeric reduction was observed for copy numbers of methanogens. Understanding the interactions between H_2 producers (e.g., bacteria, protozoa, and fungi) and consumers (e.g., methanogens and acetogens) is important to analyze the process of CH_4 production in the rumen (Cieslak *et al*., 2013). Morgavi *et al*. (2010) reported protozoa play an important role in

methanogenesis because they are considered H_2 producers with a very close physical association with methanogens, favoring the transfer of H_2 between them. Protozoa are also important in ruminal nitrogen metabolism, because they phagocyte bacteria and contribute to protein turnover and NH_3 production (Morrison and Mackie, 1996). The lack of effect of NOP on ruminal NH_3 concentration in the present study can be explained by the absence of effects on protozoa numbers (Ivan *et al*., 2001).

Reductions in CH_4 production do not always correspond to reductions in abundance of total protozoa, methanogens, or bacteria (Tekippe *et al*., 2011; Chung *et al*., 2012; Abecia *et al*., 2012). It follows that NOP may alter the function of specific microorganisms rather than the microbial community itself. Zhou *et al*. (2011) analyzed ruminal content samples from Chung *et al*. (2012) for total methanogens at various sampling times from dairy cows fed different enzyme treatments. There was no correlation between copy numbers of methanogens and CH_4 production, but some changes in the methanogen community profile were reported leading to the conclusion that particular species and metabolic activity of methanogens rather than the total methanogenic population itself may be responsible for CH_4 production.

The addition of NOP to a backgrounding diet decreased daily enteric CH_4 emissions from beef cattle without negatively affecting diet digestibility. The shift in end products of digestion (i.e., increased propionate proportion) in the rumen indicated that NOP changed microbial fermentation of feed. The small reduction (up to 5.8%) in DMI observed when NOP was fed could potentially negatively affect animal performance if the reduction in energy lost as CH_4 is not captured as ME. Further research is needed to confirm the net effects of NOP supplementation in beef cattle diets on CH_4 production and growth performance over a longer feeding period.

2.5. Literature cited

Abecia, L., Toral, P. G., Martín-García, A. I., Martínez, G., Tomkins, N. W., Molina-Alcaide, E., Newbold, C. J., and Yañez-Ruiz, D. R. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95:2027–2036.

AOAC. 2005. Official methods of analysis. 18th ed. AOAC Int., Gaithersburg MD.

AOAC. 2006. Official methods of analysis, 18th ed., revisions 1. AOAC Int., Gaithersburg, MD.

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

Burrin, D. G., and Britton, R. A. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63:888–893.

Canadian Council on Animal Care. 1993. Guide to the care and use of experimental animals. Vol. 1. 2nd ed. [E.D. Olfert, B. M. Cross, and A. A. McWilliam, editors]. CCAC, Ottawa, ON, Canada.

Chen, Y., Penner, G. B., Li, M., Oba, M., and Guan, L. L. 2011.Changes in Bacterial Diversity Associated with Epithelial Tissue in the Beef Cow Rumen during the Transition to a High-Grain Diet.

Chen, Y., Oba, M., and Guan, L. 2012. Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. Vet. Microbiol. 159:451–459.

Chung, Y.-H., Walker, N. D., McGinn, S. M., and Beauchemin, K. A. 2011. Differing effects of 2 active dried yeast (*Saccharomyces cerevisiae*) strains on ruminal acidosis and methane production in nonlactating dairy cows. J. Dairy Sci. 94:2431–2439.

Chung, Y.-H., Zhou, M., Holtshausen, L., Alexander, T. W., McAllister, T. A., Guan, L. L., Oba, M., and Beauchemin, K. A. 2012. A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. J. Dairy Sci. 95:1419–1427.

Cieslak, A., Szumacher-Strabel, M., Stochmal, A., and Oleszek, W. 2013. Plant components with specific activities against rumen methanogens. Animal 7:253–265.

Duval, S., and Kindermann, M. 2012. Use of nitooxy organic molecules in feed for reducing enteric methane emissions in ruminants, and/or to improve ruminant performance. International Patent Application WO 2012/084629 A1. World Intellectual Property Organization, Geneva, Switzerland.

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

Haisan, J., Sun, Y., Beauchemin, K., Guan, L., Duval, S., Barreda, D. R., and Oba, M. 2013. Effects of feeding 3-nitrooxypropanol, at varying levels, on methane emissions and rumen fermentation in lactating dairy cows. Adv. Anim. Biosci. 4:326 (Abstr.).

Haisan, J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R., and Oba, M. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

Hristov, A. N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J., and Oosting, S. 2013. In Mitigation of greenhouse gas emissions in livestock production – A review of technical options for non-CO_2 emissions [P. J. Gerber, B. Henderson, and H. P.

S. Makkar, editors] FAO Animal Production and Health Paper No. 177. FAO, Rome, Italy.

Ivan, M., Mir, P. S., Koening, K. M., Koening, L. M., Rode, L. M., Neill, L., Entz, T., and Mir, Z. 2001. Effects of dietary sunflower seed oil on rumen protozoa population and tissue concentration of conjugated linoleic acid in sheep. Small Rumin. Res. 41:215–227.

IPCC. 2006. Guidelines for National Greenhouse Gas Inventories. www.ipcc-nggip.iges.or.jp/public/2006gl. (Accessed 3 September 2014.).

Janssen, P. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160:1–22.

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

Li, M., Penner, G. B., Hernandez-Sanabria, E., Oba, M., and Guan, L. L. 2009. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107:1924–1934.

Martinez-Fernandez, G., Arco, A., Abecia, L., Cantalapiedra-Hijar, G., Molina-Alcaide, E., Martin-Garcia, A. I., Kindermann, M., Duval, S., and Yañez-Ruiz, D. R.. 2013. The addition of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol in the diet of sheep substantially reduces methane emissions and the effect persists over a month. Adv. Anim. Biosci. 4:368 (Abstr.).

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

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

Melendez, P., Goff, J. P., Risco, C. A., Archbald, L. F., Littell, R., and Donovan, G. A. 2004. Effect of a monensin controlled-release capsule on rumen and blood metabolites in Florida Holstein transition cows. J. Dairy Sci. 87:4182–4189.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E. and McSweeney, C. S. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108:482–491.

Morgavi, D. P., Forano, E., Martin, C., and Newbold, C. J. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4(7):1024–1036.

Morrison, M., and Mackie, R. I. 1996. Nitrogen metabolism by ruminal microorganisms: Current understanding and future perspectives. Aust. J. Agric. Res. 47:227–246.

Nagaraja, T. G., Avery, T. B., Bartley, E. E., Galitzer, S. J., and Dayton, A. D. 1981. Prevention of lactic acidosis in cattle by lasalocid or monensin. J. Anim. Sci. 53:206–216.

NRC. 1996. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

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

Penner, G. B., Beauchemin, K. A., and Mutsvangwa, T. 2006. An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. J. Dairy Sci. 89:2132–2140.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J. Dairy Sci. 97:3777–3789.

Rhine, E. D., Sims, G. K., Mulvaney, R. L., and Pratt, E. J. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Sci. Soc. Am. J. 62:473–480.

Seo, S., Tedeschi, L. O., Lanzas, C., Schwab, C. G., and Fox, D. G. 2006. Development and evaluation of empirical equations to predict feed passage rate in cattle. Anim. Feed Sci. Technol. 128:67–83.

Shima, S., and Thauer, R. K. 2005. Methyl-coenzyme M reductase and the anaerobic oxidation of methane in methanotrophic archaea. Curr. Opin. Microbiol. 8:643–648.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., and de Haan, C. 2006. Livestock's long shadow: Environmental issues and options. Food and Agriculture Organization of the United Nations, Rome, Italy.

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

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M., and Firkins, J. L. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. J. Nutr. 134:3378–3384.

Tekippe, J. A., Hristov, A. N., Heyler, K. S., Cassidy, T. W., Zheljazkov, V. D., Ferreira, J. F. S., Karnati, S. K., and Varga, G. A. 2011. Rumen fermentation and production effects of *Origanum vulgare* L. leaves in lactating dairy cows. J. Dairy Sci. 94:5065–5079.

United States Environmental Protection Agency (EPA). 2012: Global anthropogenic non-CO_2 greenhouse gas emissions: 1990–2020. www.epa.gov/climatechange/Downloads/EPAactivities/EPA_Global_NonCO_2_Projections_Dec2012.pdf. (Accessed 4 April 2014.).

Van Nevel, C., and Demeyer, D. 1995. Feed additives and other interventions for decreasing methane emissions. In Biotechnology in animal feeds and animal feeding [R. J. Wallace and A. Chesson, editors] VCH Verlagsgesellschaft mbH., Weinheim, Germany. p. 329–349.

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

Zhou, M., Chung, Y.-H., Beauchemin, K. A., Holtshausen, L., Oba, M., McAllister, T. A., and Guan, L. L. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. J. Appl. Microbiol. 111:1148–1158.

Zhou, M., Hernandez-Sanabria, E., and Guan, L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Appl. Environ. Microbiol. 75:6524–6533.

3. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet[1]

3.1. Introduction

Rumen microorganisms ferment feed and supply the ruminant host with VFA and microbial protein as fermentation products. During this process, CH_4 is produced as a byproduct and released by respiration and eructation into the atmosphere (Mitsumori and Sun, 2008; Gerber *et al.*, 2013). This represents a loss of energy to the animal ranging from 2 to 12% of GE intake (Johnson and Johnson, 1995). In addition, CH_4 is also a potent GHG with a global warming potential 28 times higher than CO_2 (IPCC, 2013). The estimated total GHG emission from livestock production is 7.1 Gt CO_2-eq per year representing 14.5% of global anthropogenic emissions (Gerber *et al*., 2013). About 44% of this total is CH_4 with beef production accounting for 41% of global livestock sector emissions and enteric fermentation as the main source (Gerber *et al*., 2013).

3-Nitrooxypropanol, developed by Duval and Kindermann (2012), is a structural analog of methyl-CoM that is believed to act as an inhibitor of the enzyme MCR during the last step of methanogenesis (Romero-Perez *et al*., 2014). It decreased CH_4 emissions *in vitro* (Romero-Perez *et al*., 2013) and *in vivo* using sheep (Martínez-Fernández *et al*., 2014), dairy cows (Haisan *et al*., 2013, 2014; Reynolds *et al*., 2014) and beef cattle (Romero-Perez *et al*., 2014). These studies report the potential of NOP to reduce CH_4 corrected for DMI from 6.7 to 59.6% depending

[1]A version of this chapter has been published. Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L.L., Oba, M., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2015. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. J. Anim.Sci. 93:1780–1791.

on the mode of supplementation; i.e., mixed with the feed, top-dressed or directly dosed into the rumen.

Overall, NOP supplementation is associated with a shift in ruminal fermentation where molar proportion of acetate is decreased and that of propionate increased with no effect on digestibility or animal productivity (Haisan *et al*., 2013, 2014; Martínez-Fernández *et al*., 2014; Reynolds *et al*., 2014; Romero-Perez *et al*., 2014). In contrast, compounds with inhibitory effect on MCR, like BES, failed to continuously decrease CH_4 production after 4 d of supplementation (Immig, 1996).

Results of short-term experiments evaluating NOP to reduce CH_4 emissions have yielded promising results; however, no studies have been conducted to evaluate its long-term effect on methanogenesis. The objective of the present study was to evaluate whether adding NOP to a beef cattle diet for 112 d resulted in a sustained reduction in enteric CH_4 emissions.

3.2. Materials and methods

The experiment was conducted at Agriculture and Agri-Food Canada's Research Centre in Lethbridge, Alberta, Canada. Cattle were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

Diet and Animal Management

A uniform group of 8 mature Angus heifers (637 ± 16.2 kg of initial BW; 21 months old) with rumen cannulas were fed a TMR daily at 1300 h (Table 3.1) consisting of 60% barley silage, 35% barley grain, and 5% vitamin–mineral supplement (DM basis). The TMR was prepared daily using a feed mixer (Data Ranger; American Calan Inc., Northwood, NH). For the

entire experiment, intake was restricted to 65% of the group average *ad libitum* DMI observed 10 d before beginning the experiment. The amount of feed supplied was calculated to slightly exceed NE required for maintenance based on the NRC recommendations (NRC, 1996). Feed restriction was necessary prevent heifers from excessive weight gain. Animals were housed in a heated research barn in individual tie stalls fitted with rubber mattresses and bedded with wood shavings. They were exercised daily for 1.5 h except when they were in the metabolic chambers.

Experimental Design and Dietary Treatments

The experiment was designed as a completely randomized design with 2 treatments: Control (0 g of NOP animal^{-1} d^{-1}; DSM Nutritional Products, AG, Kaiseraugst, Switzerland) and NOP (2 g of NOP animal^{-1} d^{-1}). The treatments were hand mixed into the TMR daily at feeding time. Animals were randomly allocated to 2 groups of 4 heifers to facilitate the measurement of CH_4 using metabolic chambers (n = 4) with two heifers in each group randomly assigned to a treatment. The duration of the experiment was 146 d, which included the following sampling periods: an initial 18-d covariate period without NOP use to establish a baseline for variables of interest; a 112-d treatment period with NOP added to the diet, divided into four 28-d intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to112); and a final 16-d recovery period in which NOP was not provided.

The NOP dose used in the present study was based on the levels used in a previous experiment (Romero-Perez *et al*., 2014) in which different doses of NOP were evaluated (0, 0.75, 2.25, and 4.50 mg/kg BW, equivalent to 0, 0.5, 1.4, and 2.8 g/d).

Table 3.1. Ingredient and chemical composition of the diet

Item	% of DM
Ingredient[1]	
Barley silage[2]	60
Barley grain, dry-rolled	35
Barley grain, ground	2.688
Calcium carbonate	1.374
Canola meal	0.500
Salt	0.158
Urea	0.110
Molasses, dried	0.108
Feedlot vitamin-mineral premix[3]	0.055
Vitamin E (500,000 IU/kg)	0.004
Flavouring agent	0.003
Chemical composition[4]	
DM	53.6 ± 2.18
OM, % of DM	92.5 ± 0.24
CP, % of DM	11.29 ± 0.63
NDF, % of DM	38.6 ± 1.35
ADF, % of DM	22.4±1.72
Starch, % of DM	33.8 ± 0.57
Fat, % of DM	1.85 ± 0.13
GE, Mcal/kg	4.9± 0.53

[1]All ingredients except barley silage and dry-rolled barley grain were provided as part of a pelleted supplement.

[2]Composition: DM, 42.4%; CP, 10.5%; NDF, 49.9%; ADF, 33.1%.

[3]Feedlot vitamin-mineral premix contained: $CaCO_3$, 35.01%; $CuSO_4$, 10.37%; $ZnSO_4$, 28.23%; Ethylenediamine dihydriodide (80% concentration),, 0.15%; selenium 1% (10,000 mg Se/kg), 5.01%; $CoSO_4$, 0.1%; $MnSO_4$, 14.54%; vitamin A (500,000,000 IU/kg), 1.71%; vitamin D (500,000,000 IU/kg), 0.17% and vitamin E (500,000 IU/kg), 4.7%.

[4]Mean ± SD; n = 6.

The amount of feed offered and refused was recorded daily for each heifer. Dry matter intake was calculated using the DM contents of the basal diet and refusal samples. If present, refusals were collected and composited by week for each animal and stored at –20°C until analyzed for DM. The basal diet and barley silage were sampled 3 times per week to monitor DM content. Dried samples of the basal diet and barley silage were pooled by sampling period or interval within the treatment period and stored for chemical analysis. Barley grain and the vitamin–mineral supplement were sampled once weekly to monitor DM content. The basal diet was adjusted when the DM content of an ingredient varied by more than 3%. Animals (not fasted) were weighed at the beginning of the experiment and before and after they went into the metabolic chambers, which corresponded to the end of each sampling period or interval within the treatment period.

Enteric Gas Production

For the last 3 d of each sampling period or interval within the treatment period, heifers were moved into 4 metabolic chambers (1 heifer/chamber) where the production of CH_4 and CO_2 was continuously monitored. The 2 groups of heifers were staggered by 1 week to allow gas measurements. Gas production was measured as described in Chapter 2.

Ruminal Variables

Starting on d 12 of the initial covariate period, d 22 of each interval within the treatment period, and d 8 of the final recovery period, ruminal pH was continuously measured for 7 d using the LRCpH logger system (Dascor, Escondido, CA). The system was standardized using buffers pH 4 and 7 at the start and end of each measurement.

The shift in millivolt reading from the electrodes between the start and end standardizations was assumed to be linear and was used to convert millivolt readings to pH units as described by Penner *et al*. (2006).

On the initial day of pH measurement for the initial covariate period and intervals within the treatment period and final recovery period, samples of rumen contents from multiple sites within the rumen (atrium, dorsal, ventral, caudodorsal, and caudoventral sacs) were collected at 0 h (prefeeding) and at 3 and 6 h after feeding. Rumen contents were strained through a PECAP polyester screen (355-µm pore size; B & S H Thompson, Ville Mont-Royal, QC, Canada) and 5 mL of the filtered ruminal fluid was added to 1 mL of 25% meta-phosphoric acid (wt/vol) for VFA determination. Another 5 mL of filtered ruminal fluid was added to 1 mL of 1% sulfuric acid (vol/vol) for NH_3 determination. These samples were immediately frozen after collection using liquid nitrogen and stored at –20°C until analyzed. Samples from whole rumen contents

collected 3 h after feeding were also prepared, frozen with liquid nitrogen, and stored at –80°C until analyzed for protozoa, methanogens, and bacteria using qPCR.

The recovery of MH was estimated from MH produced and MH utilized to produce VFA and CH_4 during rumen fermentation (Mitsumori *et al*., 2012). Calculations were done using the ruminal concentrations of acetate, propionate, butyrate, isovalerate, and valerate. Recovered MH in the form of VFA, CH_4, and H_2 was assumed to be 90% of MH produced in accordance with typicall MH recoveries observed *in vitro*. The production of H_2 was expected to be minimal for the Control treatment and was not considered in the calculation. The MH for H_2 formation during the treatment period was based on CH_4 reduction rates, which were calculated from CH_4 production in respiration chambers as proposed by Mitsumori *et al*. (2012).

Laboratory Analyses

Analyses for DM, analytical DM, OM, NDF, ADF, crude fat, GE, starch, VFA, NH_3, total bacteria, methanogens and protozoa were carried on in the same manner as indicated in Chapter 2.

Calculations and Statistical Analysis

Animal was the experimental unit for all variables. Data for ruminal pH were summarized by day for minimum, maximum, and average pH. Protozoa, methanogens, and bacteria data were not normally distributed and so a $\log_{10}$ transformation was applied before analysis with the inverse $\log_{10}$ least squares mean reported. The daily CH_4 flux was determined for each chamber and expressed relative to DMI and GE intake using intake from the CH_4 measurement day. Data were analyzed using a mixed model procedure (SAS Inst. Inc., Cary,

NC). Data were covariate adjusted for their baseline measurements. For the analysis of the treatment period, the model included the fixed effects of treatment (Control and NOP), interval within the treatment period, and their interactions. For the analysis of the recovery period, the model included the fixed effect of treatment. Group was considered a random effect. When appropriate, time, day, or interval were considered repeated measures in the model.

The variance components were estimated using the REML method and degrees of freedom were adjusted using the Kenward–Roger option. The PDIFF option was used to separate means when necessary. The covariance structure was selected based on the lowest Akaike and Bayesian information criteria values. Hourly means (at 2-h intervals) of ruminal pH and CH_4 production were compared using a *t* test to determine differences in daily pattern between treatments. Least squares means are presented and treatment differences and trends were declared significant at $P \leq 0.05$ and $P \leq 0.10$, respectively.

3.3. Results

The DMI during the experiment averaged 7.09 ± 0.15 kg and no significant effects were observed for treatment, interval, or treatment × interval interaction ($P \geq 0.11$; Table 3.2). All feed was consumed by the heifers within 4 h after feeding. Animals gained weight over the study (0.31 ± 0.05 kg/d). Minimum ruminal pH was higher for heifers fed NOP both during the treatment period ($P = 0.01$) and during the recovery period ($P = 0.01$; Table 3.2). There was no effect of treatment, interval, or treatment × interval interaction for mean, maximum, and range of pH during the treatment or recovery periods ($P \geq 0.11$). The diurnal ruminal pH pattern during the treatment period (Fig. 1) showed a consistent reduction of pH after feeding, reaching nadir 5 to 7 h later for the Control treatment and 5 h later for the NOP treatment. Subsequently, pH

Table 3.2. Dry matter intake, body weight and rumen fermentation of beef cattle fed a backgrounding diet added with 3-nitrooxypropanol

Item	Supplementation period[1]			*P*-value[2]			Recovery period[3]			
	Control	NOP	SEM	Trt	Int	Trt × Int	Control	NOP	SEM	*P*-value
DMI, kg	7.15	7.14	0.01	0.52	0.11	0.85	6.94	6.93	0.1	0.65
BW, kg	666	665	3.68	0.97	<0.01	0.55	689	686	5.63	0.78
Ruminal pH										
Minimum	5.67	5.89	0.10	0.01	0.12	0.11	5.75	5.93	0.13	0.01
Mean	6.44	6.64	0.06	0.58	0.11	0.15	6.47	6.57	0.07	0.42
Maximum	7.06	7.11	0.43	0.15	0.67	0.69	6.99	7.08	0.04	0.36
Range[4]	1.4	1.2	0.08	0.67	0.75	0.66	1.3	1.2	0.07	0.25
Total VFA, m*M*	112.6	102.7	3.8	0.12	0.04	0.5	99.5	108.1	6.3	0.36
VFA, mol/100 mol										
Acetate (A)	66.6	60.6	0.61	<0.01	0.02	0.29	67.2	65.1	0.8	0.06
Propionate (P)	17.1	20.5	0.42	<0.01	0.17	0.19	17.3	17.5	0.66	0.86
Butyrate	11.7	13.9	0.68	0.04	0.49	0.94	11.2	12.3	0.53	0.2

Valerate	1.52	2.17	0.09	<0.01	0.39	0.19	1.57	1.61	0.09	0.7
Isobutyrate	1.09	1.08	0.06	0.91	0.11	0.56	1.08	1.17	0.11	0.38
Isovalerate	1.63	2.05	0.16	0.02	0.02	0.3	1.8	2.22	0.38	0.05
A:P ratio	4.0	3.0	0.08	<0.01	0.03	0.11	3.9	3.7	0.19	0.73
NH_3, m*M*	7.7	6.2	1.02	0.2	0.14	0.85	5.5	6.4	0.67	0.33

NOP, 2 g of 3-nitrooxypropanol per animal per day.

[1]112-d period with NOP supplementation divided into four 28-d time intervals (d 1 – 28, d 29 – 56, d 57 – 84 and d 85 -112).

[2]Trt = treatment; Int = interval.

[3]16-d period without NOP supplementation.

[4]Range = maximum ruminal pH − minimum ruminal pH.

started to increase until the next feeding. Mean ruminal pH of cattle fed NOP was greater than ($P \leq 0.05$) for the Control treatment at 3, 5, 7, 9, 11, 19, 21, and 23 h after feeding (Fig. 3.1).

Total ruminal VFA was not affected by treatment during the treatment period ($P = 0.12$) or the recovery period ($P = 0.36$) and no treatment × interval interaction was observed ($P = 0.5$; Table 3.2). Additionally, an interval effect was observed for total VFA ($P = 0.04$) in which d 29 to 56 was greater compared to d 1 to 28, 57 to 84, and 85 to 112 (116.1 vs. 102.6, 106.4, and 105.5 m*M*, respectively). Molar proportions of propionate, butyrate, valerate, and isovalerate increased with NOP use during the treatment period ($P \leq 0.04$) but did not differ from the Control during the recovery period ($P \geq 0.2$) except for isovalerate, which remained greater for the NOP treatment ($P = 0.05$). There was no interval ($P \geq 0.11$) or treatment × interval interaction ($P \geq 0.19$) for propionate, butyrate, or valerate proportion; however, there was an interval effect for acetate ($P = 0.02$; 62.6, 64.2, 64.5, and 62.9 for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively) and isovalerate ($P = 0.02$; 2.1, 1.5, 1.8, and 2.0 for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively). The acetate to propionate ratio was decreased with NOP in the treatment period ($P < 0.01$) but no treatment effect was observed in the recovery period ($P = 0.33$). There was an interval effect on the A:P ratio ($P = 0.03$; 3.4, 3.6, 3.6, and 3.3 for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively) but no effect for treatment × interval interaction ($P = 0.11$). Ammonia concentration in the rumen was not affected by NOP during the treatment ($P = 0.2$) or recovery ($P = 0.33$) periods and there was no effect of interval ($P = 0.14$) or treatment × interval interaction ($P = 0.85$). Total copy number of bacterial 16S rRNA genes was not affected by NOP ($P = 0.5$), interval ($P = 0.13$), or treatment × interval interaction ($P = 0.78$) during NOP use. Total copy number of methanogenic 16S rRNA genes was decreased when NOP was offered ($P = 0.01$), but no interval ($P = 0.27$) or treatment × interval effect ($P =$

0.64) was observed. Total copy number of protozoa 18S rRNA genes was increased when NOP was offered ($P = 0.03$; Table 3.3). The treatment × interval interaction ($P = 0.85$) was not significant but an interval effect was observed ($P = 0.01$; 2.2, 4.6, 4.4, and 2.5 copies/g for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively). Total copy number for rRNA genes of these ruminal microorganisms were not affected during the recovery period ($P \geq 0.27$).

Daily CH_4 produced per animal was decreased by 59.16% when NOP was offered ($P < 0.01$) with no interval ($P = 0.19$) or treatment × interval effect ($P = 0.29$; Table 3.4). The production of CH_4 corrected for DMI was 59.21% lower for the NOP treatment compared to the Control ($P < 0.01$). Interval was not significant ($P = 0.13$) but treatment × interval interaction tended to be significant ($P = 0.06$; Fig. 2). Gross energy lost as CH_4 was decreased when NOP was offered ($P = 0.01$) and interval tended to differ ($P = 0.09$), but there was no treatment × interval interaction ($P = 0.16$). Use of NOP increased daily CO_2 produced per animal ($P = 0.01$) with no interval ($P = 0.15$) or treatment × interval interaction ($P = 0.94$).

The daily pattern of CH_4 production during the treatment period is presented in Fig. 3. For Control animals, CH_4 production rapidly increased, reaching peak production 4 h after feeding, and then a gradual reduction was observed for the rest of the day. When NOP was offered, a reduction in CH_4 emissions was observed immediately after feeding, with the lowest values observed from 2 to 4 h, and subsequently, a gradual increase was observed until prefeeding values were reached and remained for the rest of the day. Methane production of cattle fed NOP remained consistently lower at all times compared to the Control treatment.

Estimated molar proportion of MH recovered as VFA, CH_4, and H_2 during the treatment period for the Control and NOP treatments was 31.9 ± 0.7 and 38.7 ± 1.1% for VFA, 68.1 ± 0.7 and 32.0 ± 4.9% for CH_4, and 0 and 29.3 ± 4.9% for H_2, respectively. The estimated H_2

production was 35.9 ± 4.4 g/d for the NOP treatment and assumed to be absent for the Control treatment.

Table 3.3. Rumen microbes of beef cattle fed a backgrounding diet supplemented with 3-nitrooxypropanol[1]

Item	Supplementation period[2]			*P*-value[3]			Recovery period[4]			
	Control	NOP	SEM	Trt	Int	Trt × Int	Control	NOP	SEM	*P*-value
Bacteria[5]	10.77	12.08	2.22	0.5	0.13	0.78	12.69	13.63	4.6	0.82
Protozoa[6]	2.7	4.17	2.59	0.03	0.01	0.21	3.46	1.66	2.7	0.27
Methanogens[7]	15.46	6.71	0.75	<0.01	0.27	0.64	17.24	10.18	3.64	0.49

NOP, 2 g of 3-nitrooxypropanol per animal per day.

[1]Data were $\log_{10}$ transformed before statistical analysis with the inverse $\log_{10}$ least-square mean reported.

[2]112-d period with NOP supplementation divided into four 28-d time intervals (d 1 – 28, d 29 – 56, d 57 – 84 and d 85 -112).

[3]Trt = treatment; Int = interval.

[4]16-d period without NOP supplementation.

[5] $\times 10^{10}$ copy number of the 16S rRNA gene per g of rumen content.

[6] $\times 10^{5}$ copy number of the 16S rRNA gene per g of rumen content.

[7] $\times 10^{8}$ copy number of the 18S rRNA gene per g of rumen content.

Table 3.4. Effect the addition of 3-nitrooxypropanol to a backgrounding diet on enteric methane and carbon dioxide emissions of beef cattle.

Item	Supplementation period[1]			*P*-value[2]			Recovery period[3]			
	Control	NOP	SEM	Trt	Int	Trt × Int	Control	NOP	SEM	*P*-value
CH_4 emissions										
CH_4, g/d	157.93	64.49	6.79	<0.01	0.19	0.29	156.14	159.3	5.06	0.49
CH_4, g/kg DMI	22.46	9.16	0.88	<0.01	0.13	0.06	22.97	23.43	1.01	0.72
CH_4, % of GE intake[4]	6.46	2.51	0.31	<0.01	0.09	0.16	6.81	6.58	0.14	0.49
CO_2, kg/d	6.24	6.45	0.06	0.01	0.15	0.94	6.21	6.29	0.14	0.65

NOP, 2 g of 3-nitrooxypropanol per animal per day.

[1]112-d period with NOP supplementation divided into four 28-d time intervals (d 1 – 28, d 29 – 56, d 57 – 84 and d 85 -112).

[2]Trt = treatment; Int = interval.

[3]16-d period without NOP supplementation.

[4]GE intake calculated from DMI in the chambers and GE content of the TMR.

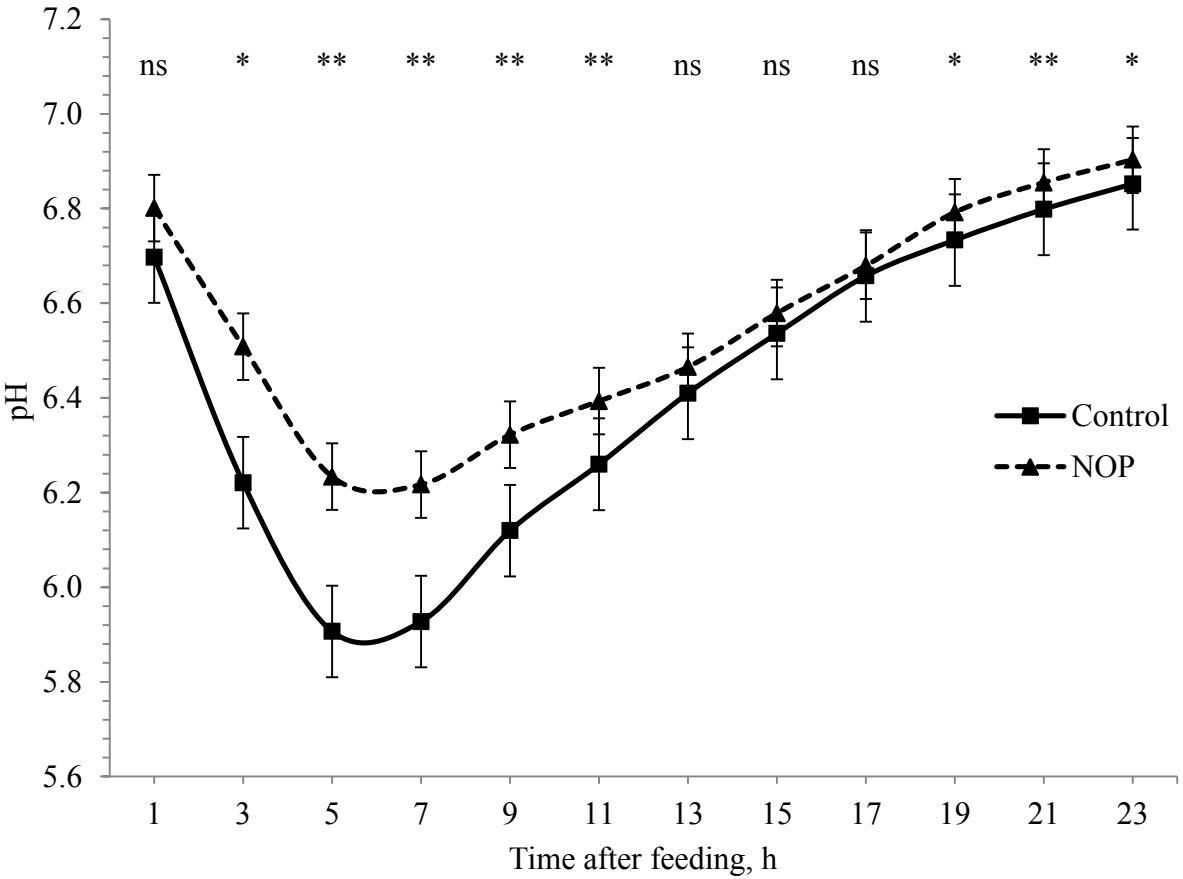

Figure 3.1. Mean daily pattern of rumen pH averaged over the treatment period from beef cattle fed a backgrounding diet with or without 2g of 3-nitrooxypropanol during periods 1 to 4. Data were summarized by 2-h intervals and means are presented. Error bars indicate the SEM. Significance of the main effect of treatment is indicated for each time point by ns, *, and, **, which correspond to not significant, $P \leq 0.05$ and $P \leq 0.01$, respectively.

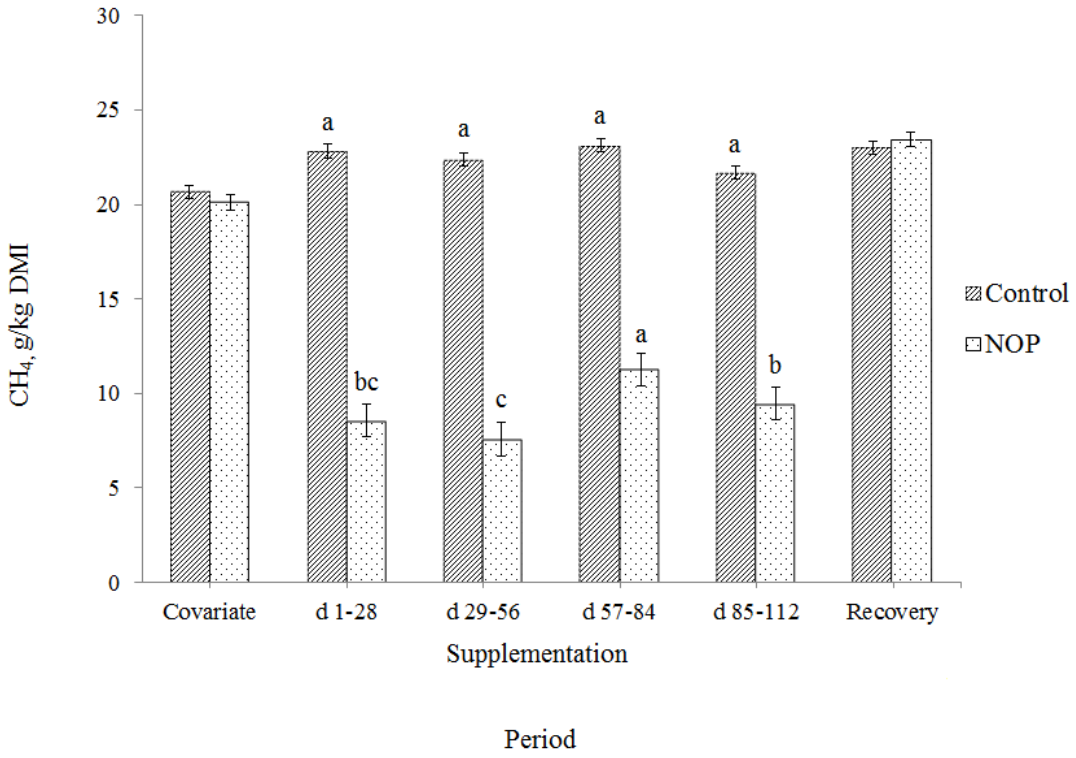

Figure 3.2. Methane production from beef cattle fed a backgrounding diet with or without 2g of 3-nitrooxypropanol. 3-Nitrooxypropanol was not supplemented during the covariate period. NOP was administered during the supplementation period which was divided into four 28-d time intervals (d 1 – 28, d 29 – 56, d 57 – 84 and d 85 -112). NOP supplementation was discontinued during the recovery period. A tendency for treatment × interval interaction was observed during supplementation period ($P = 0.06$). Within treatment, time intervals with different letter differ ($P \leq 0.05$).

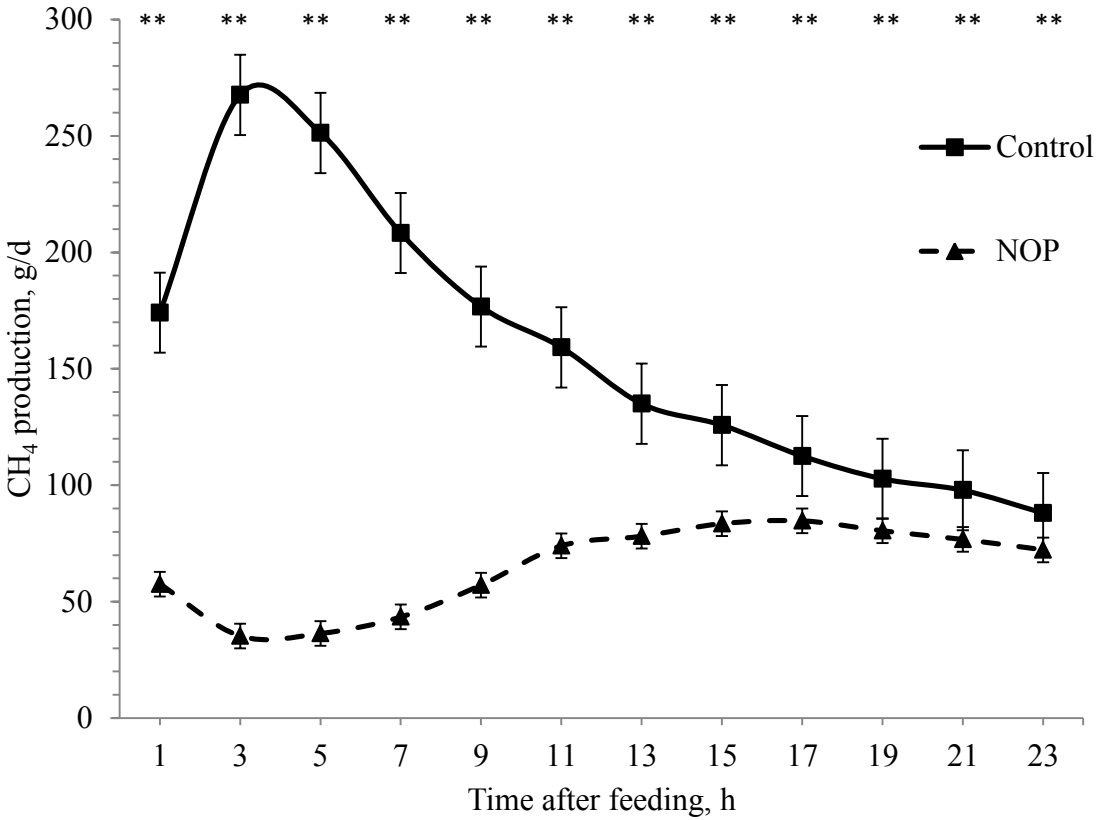

Figure 3.3. Mean daily pattern of methane production averaged over the treatment period for beef cattle fed a backgrounding diet with or without 2 g of 3-nitrooxypropanol during periods 1 to 4. Data were summarized by 2-h intervals and means are presented. Error bars indicate the SEM. Treatment significance for each time point is indicated by the symbol ** ($P \leq 0.01$).

3.4. Discussion

Total CH_4 production and CH_4 production corrected for DMI or expressed as a percentage of GE intake were decreased when NOP was offered, consistent with results from previous studies (Haisan *et al*., 2013, 2014; Martínez-Fernández *et al*., 2014; Reynolds *et al*., 2014; Romero-Perez *et al*., 2014). The magnitude of the reduction in CH_4 emissions expressed in grams/kilogram DM (59.2%) was very similar to the 59.6% reduction observed by Haisan *et al*. (2014) but was greater than the 6.7% reduction observed by Reynolds *et al*. (2014), the 25.6% reduction reported by Martínez-Fernández *et al*. (2014), and the 33.1% reduction reported by Romero-Perez *et al*. (2014). In both the present study and the study by Haisan *et al*. (2014), NOP was mixed with the diet, which allowed NOP to be gradually introduced into the rumen as the animals consumed the feed. This synchronization between gradual NOP delivery into the rumen and feed fermentation may have improved the inhibitory potential of NOP. Synchronization between feed digestion and NOP consumption does not occur when NOP is dosed directly into the rumen through the rumen cannula as was the method used by Martínez-Fernández *et al*. (2014) and Reynolds *et al*. (2014) and only to a minor extent when NOP is top-dressed onto the feed as was done by Romero-Perez *et al*. (2014). Mixing NOP with feed appears to be an effective means of providing the compound to cattle. Although pure NOP is a volatile compound that becomes less stable with increasing temperature, the diluted formulation used herein (10% NOP in silicon dioxide) was effective in maintaining its CH_4 reducing potential. The assertion that the method of providing NOP to the animal has an effect on its response is made further supported by comparing the amount of NOP provided per kilogram of DMI in the present study (284 mg NOP/kg DMI) with a previous study using beef cattle fed a similar diet (345 mg NOP/kg DMI; Romero-Perez *et al*., 2014), relative to the reductions in CH_4 achieved. The results

indicate that even when supplementing greater doses of NOP adjusted for DMI, the reduction in CH_4 emissions was smaller (33.0%; Romero-Perez *et al*., 2014) than in the present study (59.2%). The restricted amount of feed offered herein (7.04 ± 0.27 kg) could have increased the time that NOP remained in the rumen as a consequence of a decreased rate of passage. Dilution rate of the liquid phase is positively affected by DMI (Seo *et al*., 2006) wherein NOP is expected to be present (Romero-Perez *et al*., 2014). However, even when supplementing only 129 mg NOP/kg DMI to dairy cows consuming 19.3 kg DM/d (Haisan *et al*., 2014), where a fast rate of passage would be expected, the observed reduction in CH_4 production was substantial (59.6%).

The daily CH_4 production profile in the present experiment differs from that reported by Reynolds *et al*. (2014) and Romero-Perez *et al*. (2014). The transitory effect observed in those studies was not present herein; in contrast, the reduction in CH_4 emissions was more prolonged and pronounced over the day in the present study. This suggest that the mode of supplementation (i.e., mixed with the feed, top-dressed, or dosed directly into the rumen) is an important factor to consider when using NOP. Gradual consumption of NOP and the synchronization of NOP and feed digestion achieved by mixing NOP with the feed could potentially improve the concentration of NOP in the rumen over time, especially at the time of maximum fermentation.

The treatment × interval interaction tended to be significant for CH_4 production (Fig. 2) and was caused by a greater CH_4 production with the NOP treatment during d 57 to 84 compared with d 1 to 28 and 29 to 56 ($P \leq 0.03$). However, the later reduction in CH_4 emissions during d 85 to 112 to a level similar to d 1 to 28 for the NOP treatment ($P = 0.14$) suggests there was no adaptation of animals to NOP over time. The inhibitory effect of NOP on CH_4 emissions was lost once the supplementation was stopped during the recovery period. *In vitro* continuous culture studies done in our lab (A. Romero-Perez, E. K. Okine, L. L. Guan, S. M. Duval, M.

Kindermann, and K. A. Beauchemin, unpublished data) where CH_4 production was decreased by 71% with NOP addition, showed that within 3 d after supplementation was discontinued, CH_4 inhibition was decreased on average 12% units per day (i.e., from 71% reduction to 35% reduction). This suggests that *in vivo* CH_4 production may be restored to baseline levelswithin less than 1 week after withdrawal of NOP. Previous *in vivo* studies using enzymatic inhibitors of methanogenesis showed adaptation (i.e., lack of CH_4 inhibition) after short periods of supplementation. The supplementation of BES to sheep decreased CH_4 concentration in the rumen from 40% to less than 1%; however, after 4 d of administration, the rumen ecosystem adapted to BES and CH_4 concentration in the rumen increased to 20% (Immig *et al*., 1996). When Knight *et al*. (2011) supplemented nonlactating dairy cows with chloroform, a known CH_4 inhibitor, they observed a drastic reduction in CH_4 emissions after 1 week of supplementation but afterward CH_4 production gradually increased such that there were no differences between control and treated cows by d 39 of supplementation. Tomkins *et al*. (2009) supplemented beef cattle on a long-term basis with BCM, a halogenated CH_4 analog with inhibitory effect on methanogenesis, and decreased CH_4 production by 60, 35, and 40% on d 30, 60, and 90, respectively. Although CH_4 production was decreased after 90 d of providing BCM, this compound cannot be recommended for commercial use because uncomplexed BCM has an ozone-depleting effect (Tomkins *et al*., 2009). The use of synthetic compounds such as NOP in animal production will depend on the ease of registration by regulatory officials within a particular country or region. For example, use of monensin as a feed additive to improve growth and milk production in beef and dairy cattle was not permitted in some countries (EMEA, 2007); however, it is used extensively in Canada, the United States, Mexico, Australia, and New Zealand (Duffield *et al*., 2008). Additionally, the practical implementation of NOP as a CH_4

mitigation strategy under more extensive conditions, such as grazing animals, will depend on development of stable NOP formulations in a range of different environments and technologies to dose NOP to pastured cattle.

Carbon dioxide is a substrate for CH_4 formation; therefore, inhibition of CH_4 production can theoretically increase CO_2 escape from the rumen, as was observed in the present experiment and by Romero-Perez *et al*. (2014). An increase in ruminal fermentation can also increase CO_2 production; however, this is less probable herein because NOP has been reported to slightly reduce VFA concentration in the rumen (Romero-Perez *et al*., 2014) without affecting total tract digestibility (Reynolds *et al*., 2014; Romero-Perez *et al*., 2014). Such reduction in VFA concentration without effect on total tract digestibility could be explained by greater postruminal digestion or by an increased rate of VFA absorption or passage from the rumen. Although CO_2 was increased in the present experiment, this increment was more than offset by the reduction in CH_4, when calculated based on CO_2 equivalents. Additionally, for GHG inventory, the Intergovernmental Panel on Climate Change (2006) assumes that the carbon released by animals as CO_2 during respiration is zero because CO_2 photosynthesized by plants is returned to the atmosphere as respired CO_2.

The increase in minimum ruminal pH is consistent with previous studies in which NOP was offered (Reynolds *et al*., 2014; Romero-Perez *et al*., 2014) and coincides with a reduction in total VFA concentration in the rumen. In the present study, a numerical reduction of total VFA concentration when NOP was offered was also observed. The diurnal ruminal pH pattern observed herein is similar to that reported by Romero-Perez *et al*. (2014), whereby a consistent reduction of pH occurred after feeding, reaching a nadir 7 to 9 h later, with the lowest pH values ranging from approximately 6.2 to 6.4 for the Control and NOP (4.5 mg/kg BW) treatments,

respectively. In the present experiment, the reduction in pH was more severe, reaching nadir 6 h after feeding, with values ranging from approximately 5.8 to 6.2 for the Control and NOP treatments, respectively. The diet used in the present experiment was similar to that used by Romero-Perez *et al*. (2014), and therefore, the greater reduction in pH in this experiment can be related to the short time that animals expended to consume the entire feed allotment (approximately 4 h). This consumption behavior can be related to the feed restriction to which animals were subjected (Munksgaard *et al*., 2005). Consequently, more feed reached the rumen in a shorter time, increasing fermentation and VFA production in the rumen with a rapid reduction in pH.

The molar proportions of individual VFA were affected by inclusion of NOP in the diet. Providing NOP to cattle is proven to reduce acetate proportion, increase propionate and butyrate proportions, and reduce the acetate to propionate ratio (Martínez-Fernández *et al*., 2014; Reynolds *et al*., 2014; Romero-Perez *et al*., 2014). Both CH_4 and propionate production in the rumen are net sinks for H_2. When methanogenesis is inhibited, propionate production is a common alternative route for MH disposal. However, when CH_4 production is inhibited by using enzymatic inhibitors, it is also possible that MH not used for CH4 formation simply escapes from the rumen without increasing propionate production (Mitsumori *et al*., 2012; Leng, 2014). The molar proportion of valerate and isovalerate were higher for the NOP treatment whereas that for isobutyrate remained unchanged. The increase in isovalerate proportion suggests that NOP can affect the deamination process in the rumen; this is because deamination of leucine is expected to result in CO_2, NH_3, and isovalerate (Russell, 2002). Adding NOP to the diet had no effect on ruminal NH_3 concentration, which may be related to very low concentrations of isovalerate. The increase in molar proportion of valerate observed in the present study can be explained on the

basis that valerate can be a net sink for MH (Russell, 2002); however, due to its relatively low concentration in the rumen, this pathway may be of minor importance for MH disposal (Hristov *et al*., 2013).

As previously mentioned, a reduction in CH_4 production can increase the release of CO_2 from the rumen but may also increase H_2 emissions if not efficiently redirected to other metabolic pathways, such as propionate production. In the present study the proportion of MH captured in CH_4 was decreased by 36.1% units (i.e., from 68.1% for the Control treatment to 32.0% for the NOP treatment) and that for VFA was increased by 6.8% units (i.e., from 31.9% for the Control treatment to 38.7% for the NOP treatment). The remaining MH (29.3% units) not used for CH_4 production when NOP was added to the diet was assumed to be used for H_2 production (35.9 ± 4.4 g/d). The H_2 production obtained with the MH balance calculation in the present study is an estimation and needs further verification by direct H_2 measurements. We have observed that H_2 production and concentration in the gas mixture were increased with NOP added to feed when using Rusitec fermenters (A. Romero-Perez, E. K. Okine, L. L. Guan, S. M. Duval, M. Kindermann, and K. A. Beauchemin, unpublished data). This H_2 increase has also been observed *in vivo* when other CH_4 inhibitors such as BCM (Mitsumori *et al*., 2012) or BES (Immig *et al*., 1996) were used. Surprisingly, total tract digestibility in dairy cows fed diets containing NOP (51.2% forage diet; Reynolds *et al*., 2014), beef cattle fed NOP (60% forage diet; Romero-Perez *et al*., 2014), and goats fed BCM (50% forage diet; Mitsumori *et al*., 2012) has not been negatively affected. Additionally, no effect was observed for milk (Haisan *et al*., 2013, 2014; Reynolds *et al*., 2014; Romero-Perez *et al*., 2014) or meat production (Mitsumori *et al*., 2012; Romero-Perez *et al*., 2014), contrary to the expectation that an increase in H_2 partial pressure would result in a negative feedback effect on the regeneration of reduced cofactors (i.e.,

NADH and the reduced form of nicotinamide adenine dinucleotide phosphate) and, consequently, on feed digestion and animal production. Leng (2014) proposed that if formate is produced within the biofilm and then released to the external fluid and transformed to H_2 and CO_2, the partial pressure of H_2 within the biofilm would remain low, allowing for the oxidation of reduced cofactors and fermentation to continue.

Effects of NOP on the microbial community reported in the literature are inconsistent. Martínez-Fernández *et al*. (2014), Haisan *et al*. (2013), and Romero-Perez *et al*. (2014) observed no changes in total copy number of 16S and 18S rRNA genes from different microbes (bacteria, methanogens, and/or protozoa) when providing different doses of NOP to sheep or dairy or beef cattle, respectively, whereas Haisan *et al*. (2014) reported a reduction in total copy number of 16S rRNA genes of methanogens. The reduction in CH_4 emissions without a direct effect on total number of methanogens has been explained on the basis that NOP could possibly affect the activity of individual species rather that the total number of methanogens, as previously observed (Zhou *et al*., 2011). However, under *in vivo* conditions in which the provision of NOP drastically decreased (59.6%) CH_4 emissions (Haisan *et al*., 2014), a tendency for a positive relationship between number of methanogens and CH_4 production was observed. In the present study, the 59.2% reduction in CH_4 emissions (g CH_4/kg of DMI) was consistent with the 56.6% reduction in total copy number of methanogens and suggests that a reduction of total copy number of methanogens when NOP is offered may occur when there is a relatively greater CH_4 mitigation effect of NOP. Although the various experiments evaluating NOP have reported reductions in CH_4 emissions, reasons for the inconsistent effects on rumen microorganisms, especially the methanogens, is unknown. It is possible that not all individuals within a herd respond in the same manner to NOP and the possibility that some animals may exhibit adaptation over time cannot be

discounted. When evaluating the effects of providing goats with BCM, Mitsumori *et al*. (2012) observed increases up to 6-fold in *Prevotella* spp. In that study, an increase in H_2 production together with an increase in propionate concentration was reported with increasing doses of BCM, and it was proposed that the rumen adapted to increased H_2 levels by shifting fermentation to propionate via *Prevotella* spp. In the current study, the observed increase in propionate molar proportion when NOP was provided indicates a redirection of MH in the rumen and suggests the need to evaluate individual species of ruminal bacteria when NOP is added to cattle diets. Rumen methanogens living in association with protozoa are responsible for 9 to 25% of methanogenesis in rumen fluid (Newbold *et al*., 1995). Protozoa are H_2 producers and have a symbiotic relationship with methanogens conducting interspecies H_2 transfer (Vogels *et al*., 1980; Finlay *et al*., 1994; Morgavi *et al*., 2010). Consequently, defaunation has been proposed as a mitigation strategy with a potential to reduce CH_4 emissions by about 10.5% (Morgavi *et al*., 2010). Total copy number of protozoa in the present study was increased with NOP use; however, methanogenesis and copy number of methanogens were decreased. Soliva *et al*. (2011) evaluated the synthetic compound 3-azidopropionic acid ethyl ester (APEE), a structural analog of methyl-CoM, using Rusitec fermenters. They observed a drastic reduction in CH_4 emissions (98%) together with a 3-fold increase in entodiniomorph protozoal number and a 7.5-fold increase in H_2 production. Soliva *et al*. (2011) suggested that the additional H_2 supplied by these protozoa did not compensate for the adverse action of APEE on methanogenesis. The mechanism whereby rumen protozoa copy number increased when NOP was added to the diet in the present study is not clear and requires further investigation.

Most of the ruminal fermentation variables evaluated during the recovery period were not different between the Control and NOP treatments, except for minimum pH and acetate and

isovalerate proportions. The lack of effect during the recovery period for most ruminal fermentation variables is in agreement with the lack of effect on CH_4 emissions during this period. The recovery for CH_4 production seems to be faster than the recovery for certain VFA such as acetate or isovalerate and suggests that methanogens may adapt faster to new ruminal conditions after NOP provision is discontinued than microorganisms producing acetate and isovalerate.

Adding NOP to a backgrounding diet by mixing it into the TMR at the time of feeding decreased CH_4 emissions per kilogram of DMI by 59.2% over 112 d with no signs of adaptation. Rumen fermentation was affected by the addition of NOP. Most notably, minimum pH was increased, molar proportion of acetate was decreased, and molar proportion of propionate was increased in cattle fed NOP indicating a redirection of H_2 to propionate formation. The reduction of total number of methanogens is in agreement with the reduction in CH_4 emissions and suggests that a substantial reduction in CH_4 emissions with NOP addition to diets may partially occur as a result of a decreased methanogen population. Residual effects of NOP on the variables studied were either nonexistent or minimal during the recovery period (16 d) when supplementation was discontinued. The synchronization of NOP availability in the rumen and feed digestion achieved by mixing NOP with the feed can potentially improve the synchronization between NOP concentration in the rumen and rumen fermentation, thereby maximizing the CH_4 inhibitory potential of NOP.

3.5. Literature cited

AOAC. 2005. Official methods of analysis. 18th ed. AOAC Int., Gaithersburg MD.

AOAC. 2006. Official Methods of Analysis, 18th ed., revisions 1. AOAC International, Gaithersburg, MD, USA.

Canadian Council on Animal Care. 2009. CCAC guidelines on: The care and use of farm animals in research, teaching and testing. Ottawa, ON, Canada. http://www.ccac.ca/Documents/Standards/Guidelines/Farm_Animals.pdf. (Accessed February 10, 2015.)

Chung, Y. -H., Walker, N. D., McGinn, S. M., and Beauchemin, K. A. 2011. Differing effects of 2 active dried yeast (Saccharomyces cerevisiae) strains on ruminal acidosis and methane production in nonlactating dairy cows. J. Dairy Sci. 94:2431–2439.

Duffield, T. F., Rabiee, A. R., and Lean, I. J. 2008. A Meta-Analysis of the Impact of Monensin in Lactating Dairy Cattle. Part 2. Production Effects. J. Dairy Sci. 91:1347–1360.

Duval, S., and Kindermann, M. 2012. Use of nitrooxy organic molecules in feed for reducing enteric methnae emsions in ruminants, and/or to improve ruminant performance, International Patent Application WO 2012/084629 A1, World Intellectual Property Organization. Geneve, Switzerland.

European Medicines Agency (EMEA). 2007. Monensin (Cattle, including dairy cows). EMEA/CVMP/185123/2007-Final. EMEA, London UK.

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M., and Hirt, R. P. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiology Letters 117:157–162.

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

Haisan, J., Sun, Y., Beauchemin, K., Guan, L., Duval, S., Barreda, D. R., and Oba, M. 2013. Effects of feeding 3-nitrooxypropanol, at varying levels, on methane emissions and rumen fermentation in lactating dairy cows. Adv. Anim. Biosci. 4:326 (Abstr.).

Haisan J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R., and Oba, M. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

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

Immig, I., Demeyer, D., Fiedler, D., Van Nevel, C., and Mbanzamihigo, L. 1996. Attempts to induce reductive acetogenesis into a sheep rumen. Archiv.für Tierernaehrung. 49:4, 363–370.

IPCC. 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y.

Xia, V. Bex and P.M. Midgley, editors]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

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

Knight, T., Ronimus, R.S., Dey, D., Tootill, C., Naylor, G., Evans, P., Molano, G., Smith, A., Tavendale, M., Pinares-Patiño, C.S., and Clark, H. 2011. Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. Anim. Feed Sci. and Tech. 166–167:101–112.

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci.54:519–543.

Li, M., Penner, G. B., Hernandez-Sanabria, E., Oba, M., and Guan, L. L. 2009. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107:1924–1934.

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

Mitsumori, M. and Sun, W. 2008. Control of rumen microbial fermentation for mitigating methane emissions from the rumen. Asian-Aust. J. Anim. Sci. 1(vol. 21):144-154.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., and McSweeney, C. S. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. British Journal of Nutrition 108:482–491.

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

Munksgaard, L., Jensen, M. B., Pedersen, L. J., Hansen, S. W., and Matthews, L.. 2005. Quantifying behavioural priorities—Effects of time constraints on behaviour of dairy cows, *Bos taurus*. Appl. Anim. Behav. Sci. 92:3–14.

Newbold, C. J., Lassalasl, B., and Jouany, J.P. 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production *in vitro*. Lett. Appl. Microbiol. 21:230–234.

NRC. 1996. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Penner, G. B., Beauchemin, K. A., and Mutsvangwa, T. 2006. An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. J. Dairy Sci. 89:2132-2140.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of incremental rumen doses of 3-nitrooxypropanol on methane production, digestion and rumen parameters in lactating dairy cows. J. Dairy Sci. 97:3777–3789.

Rhine, E. D., Sims, G. K., Mulvaney, R. L., and Pratt, E. J.. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Sci. Soc. Am. J. 62:473–480.

Romero-Perez, A., Beauchemin, K. A., Okine, E. K., and Duval, S. M. 2013. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). Adv. Anim. Biosci. 4:389 (Abstr.).

Romero-Perez A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., and Beauchemin, K. A. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J Anim. Sci. 92:1–12. doi:10.2527/jas2014-7573.

Russell, J. B. 2002. Rumen microbiology and its role on ruminant nutrition. J. B. Russell, Ithaka, NY.

Seo, S., Tedeschi, L. O., Lanzas, C., Schwab, C. G., and Fox, D. G. 2006. Development and evaluation of empirical equations to predict feed passage rate in cattle. Anim. Feed Sci. Technol. 128:67–83.

Soliva, C. R., Amelchanka, S. L., Duval, S. M., and Kreuzer, M. 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). Br. J. Nutr. 106:114-122.

Tomkins, N. W., Colegate, S. M. and Hunter, R. A. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49:1053–1058.

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

Vogels, G. D., Hoppe, W. F. and Stumm, C. K. 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40:608-612.

Zhou M., Chung, Y. -H., Beauchemin, K. A., Holtshausen, L., Oba, M., McAllister, T. A., and Guan, L. L. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. J. Appl. Microbiol. 111:1148–1158.

4. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec)[1]

4.1. Introduction

Methane is a GHG with a global warming potential 28 times greater than CO_2 over a 100 yr time horizon (IPCC, 2013). Enteric fermentation of feed, mainly from ruminant livestock, contributes to 17 and 3.3% of global CH_4 and total GHG emissions, respectively (Knapp *et al*., 2014). About 6% of the ingested energy by cattle is lost as eructated CH_4 (Johnson and Johnson, 1995). Different strategies have been proposed to reduce CH_4 emissions from animal agriculture. Currently, improving forage quality, optimizing rumen function for higher microbial protein synthesis through feeding balanced diets, and enhancing the overall efficiency of dietary nutrient use are the most common means of decreasing CH_4 emissions per unit of animal product (Hristov *et al*., 2013). Additionally, the use of CH_4 inhibitors could become one of the most effective strategies to mitigate CH_4 production as they commonly have a high mitigation potential (i.e., more than 30% reduction) and typically do not negatively affect animal feed intake or productivity (Hristov *et al*., 2013). However, these compounds are not recommended at this time for use at the farm level because they have not been proven to be safe for animals or the environment over the long term.

Enteric CH_4 is produced by methanogenic Archaea, which utilizes MCR to catalyze the reduction of methyl-CoM with CoB to CH_4 and heterodisulfide during the last step of

[1]A version of this chapter has been published. Romero-Pérez, A., Okine, E. K., Guan, L. L., Duval, S. M., Kindermann, M., and Beauchemin, K. A., 2015. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). Anim. Feed Sci. Technol. 209:98–109.

methanogenesis (Shima and Thauer, 2005). The enzyme MCR possesses a prostetic group (F_{430}) composed of two moles of a nickel porphinoid, which can traverse the oxidation states I, II, and III of Ni, but must be in the Ni(I) form to be active (Thauer, 1998). Duval and Kindermann (2012) developed NOP, a CH_4 inhibitor with a chemical structure similar to methyl-CoM, which inhibits methanogenesis by quenching the active form of MCR via a radical type mechanism in which nitrite is released as a by-product (Prakash, 2014). Beef cattle fed NOP have not shown adaptation in CH_4 response with long-term feeding (Romero-Perez *et al*., 2015). The evaluation of NOP over a range of experimental procedures including *in vitro* batch cultures, sheep (Martínez-Fernández *et al*., 2014), beef (Romero-Perez *et al*., 2014, 2015) and dairy cattle (Haisan *et al*., 2014; Reynolds *et al*., 2014) indicate consistent reductions in CH_4 production. However, the magnitude of this reduction varies with factors such as dose, diet, method of supplementation and animal type. Typically NOP addition has changed rumen fermentation parameters by decreasing acetate and increasing propionate concentration (Haisan *et al*., 2014; Martínez-Fernández *et al*., 2014; Romero-Perez *et al*., 2014, 2015), possibly due to propionate being the most important sink of H_2 in the rumen after CH_4 (McAllister and Newbold, 2008). However, the decrease in CH_4 production is not completely explained in stoichiometric terms by moderate increases in propionate concentration. Indeed, the use of specific CH_4 inhibitors has been related to H_2 accumulation both *in vitro* (Immig *et al*., 1996; Soliva *et al*., 2011; O'Brien *et al*., 2014) and *in vivo* using direct measurement (Immig *et al*., 1996; Mitsumori *et al*., 2012), or stoichiometric calculations (Mitsumori *et al*., 2012; Romero-Perez *et al*., 2015). Thus, we hypothesized that accumulation of excess H_2 would also occur with the addition of NOP to the Rusitec. The objective of the present experiment was to evaluate effects of dose of NOP using

the Rusitec system on rumen fermentation parameters, digestibility, gas production, microbial community profile, microbial protein synthesis and MH balance.

4.2. Materials and methods

The experiment was conducted at Agriculture and Agri-Food Canada's Research Centre in Lethbridge, AB, Canada. Animals used as rumen content donors were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

Experimental design, treatments, and diet

The experiment was arranged as a completely randomized block design with 2 blocks (Rusitec apparatuses) and 4 treatments. Treatments were NOP [DSM Nutritional Products Ltd., Kaiseraugst, Switzerland; 50% NOP on silicon dioxide (SiO_2)], supplied at 0, 5, 10 and 20 mg of active compound $vessel^{-1}$ d^{-1}. Rationale for the levels of NOP chosen was based on the highest dose of NOP used in a previous *in vivo* experiment (333 mg of NOP/kg of DMI; Romero-Perez *et al*., 2014), with the theoretical dilution of NOP in the rumen being calculated based on estimated rumen capacity according to Nutt *et al*. (1980).

The diet fed to Rusitec consisted of 6 g of barley silage, 3.5 g of barley grain and 0.5 g of supplement (DM basis; Table 4.1). The diet was dried and ground through a 4-mm screen. Samples (10 g) were weighed into pre-labeled nylon bags (7 × 12 cm; 51 μm mesh opening; Sefar America Inc., New York, USA), heat-sealed and stored at room temperature until utilized.

Table 4.1. Ingredients and chemical composition of the basal diet fed to Rusitec.

Item	g/kg of DM
Ingredient[1]	
Barley silage	600
Barley grain, dry-rolled	350
Barley grain, ground	26.9
Calcium carbonate	13.7
Canola meal	5.00
Salt	1.58
Urea	1.10
Molasses, dried	1.08
Vitamin-mineral premix[2]	0.55
Vitamin E (500,000 IU/kg)	0.04
Flavouring agent	0.03
Chemical composition[3]	
DM[4]	921
OM	929
CP	105
NDF	361
ADF	217

[1]All ingredients except barley silage and dry-rolled barley grain were part of the supplement.

[2]Vitamin-mineral premix contained (g/kg of DM): $CaCO_3$, 350.1; CuSO4, 103.7; ZnSO4, 282.3; ethylene diaminedihydriodide (800 g/kg), 1.5; selenium (10 g Se/kg), 50.1; $CoSO_4$, 1.0; $MnSO_4$, 145.4; vitamin A (500,000,000 IU/kg), 17.1; vitamin D (500,000,000 IU/kg), 1.7; and vitamin E (500,000 IU/kg), 47.0.

[3]DM = Dry matter; OM = Organic matter; CP = Crude protein; NDF = Neutral detergent fibre; ADF = Acid detergent fibre.

[4]Expressed as g/kg of feed

Rumen simulation technique

Rumen fluid and solid digesta used in the experiment was obtained 2 h after the morning feeding from 4 rumen fistulated cattle fed a high-forage diet containing 850 g/kg of barley silage, 120 g/kg of barley grain and 30 g/kg of mineral-vitamin supplement on a DM basis. Rumen fluid was separated from rumen contents by filtration through four layers of cheesecloth, and then pooled into insulated thermos where pH was measured. In addition, approximately 160 g of solid rumen content were also collected for initial inoculation of the fermenters.

Artificial saliva (McDougall, 1948) was prepared according to Martínez *et al*. (2009) and modified to contain $(NH_4)_2SO_4$ (pH = 8.2; 9.8g/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 $(NH_4)_2SO_4$). Saliva was administered at a renewal rate of 2.9% h^{-1}. Fresh artificial saliva was prepared daily throughout the experiment.

Two Rusitec apparatuses (Czerkawski and Breckenridge, 1977) each equipped with eight 1-L volume anaerobic fermenters were used in this study. Each fermenter was outfitted with a port for infusion of artificial saliva and collection of effluent. On the first day of the experiment, each fermenter was filled with a mixture containing 20% of artificial saliva and 80% strained rumen fluid. Two pre-labeled nylon bags, one containing 10 g of solid rumen digesta (wet weight) and the other containing 10 g (DM) of the diet, were also allocated into each fermenter. After 24 h, the nylon bag containing solid rumen digesta was removed and replaced by a nylon bag containing 10 g of the mixed diet. Thereafter, one bag was replaced daily around 0900 h so that each bag remained in the fermenter for 48 h except for the last day of the experiment when one bag in each vessel was removed after 24 h. Collectively, the fermenters were immersed in a water bath maintained constantly at 39ºC. Effluent was collected in 1-L Erlenmeyer flasks which in turn were connected to 2-L plastic bags for gas collection. At the time of the daily feed bag exchange, rumen fluid pH, total gas production, and effluent volume from each fermenter were measured. Starting at d 8, different treatments of NOP (powder formulation) were mixed with the diet contained in each feed bags. During feed bag exchange, the fermenters were flushed with O_2-free CO_2 to maintain anaerobiosis.

Dry matter and organic matter disappearance

Dry matter (DMD) and OM disappearance (OMD) at 48 h were determined on d 9, 10, 11, 12, and 13 of the experimental period. Feed bags were withdrawn from each fermenter and washed gently under cold running water until the effluent was clear. The bags were then dried at 55ºC for 48 h for analysis of DM. The residues were pooled over the 5 d and ground in a Wiley mill (A.H. Thomas, Philadelphia, PA, USA) through a 1-mm screen and stored until analyzed for OM.

Fermentation gases and end-products

Total gas production was determined daily throughout the duration of the experiment using a gas meter (Alexander-Wright, London). From d 9 to 13, before gas volume measurement, a gas sample (20 mL) was collected directly from each bag using a 20 mL syringe and injected into evacuated 6.8 mL Exetainer vials (Labco Ltd., Wycombe, Bucks, UK). Gas samples were stored at room temperature until the end of the experiment and subsequently analysed for CH_4 and H_2.

The effluent volume was regularly visually monitored to ensure the inflow of artificial saliva in the fermenters closely matched outflow. The total volume of the effluent was determined on a daily basis at the time of feeding. Fermentation liquid (5 mL) was collected directly from each fermentation vessel and placed in 7 mL screw capped vials containing 1 mL of phosphoric acid (250 g/L) or 1 mL of H_2SO_4 (10 mL/L) for VFA or NH_3 analysis respectively. Samples were stored at -20°C until analyzed.

Microbial community

Samples for protozoa counting were obtained on d 9 to 13 of the trial. At the time of feed bag exchange, bags with feed residuals after 24 and 48 h incubation were squeezed by hand to expel the excess liquid and 2.5 mL of the fluid obtained was added to 2.5 mL of methyl green-formalin-saline solution (Ogimoto and Imai, 1981) and stored in a dark place at room temperature until enumeration of protozoa.

On d 14, feed residuals after 48 h digestion and samples from the liquid phase were collected for analysis of total copy number of 16S rRNA gene for bacteria and methanogens. Total feed residuals were deposited in 50 mL Falcon tubes and samples from the liquid phase (5 mL) were collected directly from each vessel and deposited in 7 mL screw capped vials. Both

solid and liquid samples were immediately frozen using liquid nitrogen and stored at -80°C until analysis.

Microbial protein synthesis

Starting on d 9 until the end of the experiment, bacteria in the fermenters were labeled with ^{15}N by replacing $(NH_4)_2SO_4$ in the artificial saliva with ^{15}N-enriched $(NH_4)_2SO_4$ (10 atom % ^{15}N). On d 15, 250 mL of the 24 h effluent preserved with 4 mL of sodium azide (200 g/L) was weighed, centrifuged (20,000 g; 30 min; 4°C) and the supernatant was discarded while the pellet was retained. To obtain the liquid-associated microorganisms (LAM), the remaining effluent was centrifuged (500 g; 10 min; 4°C) to remove feed particles. The supernatant was recovered, centrifuged again (20,000 g for 30 min at 4°C), the supernatant discarded and pellet retained. The solid-associated microorganisms (SAM) were obtained from feed residues in the bags. Briefly, bags with feed residuals after 24 and 48 h of digestion were removed from the fermenter, gently squeezed, weighed, and mixed together. About 4 g of residuals were sampled and stored at -40°C until determination of N and ^{15}N enrichment. For SAM isolation, the remaining residuals were placed directly into stomacher bags, 50 mL of artificial saliva were added and samples were processed twice for 1 min in a Stomacher 400 laboratory blender (Seward Medical Ltd., London, UK). Finally, to obtain SAM pellets, the processed residues were squeezed through four layers of cheesecloth and the liquid obtained centrifuged as described above for LAM. The final pellets obtained in previous processes were re-suspended with a minimum amount of distilled water and stored at -40°C until analysed for N and ^{15}N enrichment. Feed substrate was also analyzed for its natural ^{15}N content, and this value was used for background correction before ^{15}N infusion.

Analytical procedures

Analyses were performed on each sample in duplicate; when the coefficient of variation was greater than 5%, the analysis was repeated. Samples of dried feed and feed residuals were ground in a Wiley mill (A.H. Thomas, Philadelphia, PA, USA) through a 1-mm screen. Analytical DM was determined by drying at 135°C for 2 h (AOAC, 2006; Method 930.15) followed by hot weighing. The OM was calculated as the difference between DM and ash (AOAC, 2006; Method 942.05). The NDF and ADF contents were determined according to Van Soest *et al*. (1991) with heat-stable amylase and sodium sulfite used in the NDF procedure. Re-suspended pellets and feed residues for microbial protein synthesis (MPS) analysis were freeze dried and ball ground (Mixer Mill MM2000; Retsch, Haan, Germany) before determination of N and ^{15}N enrichment. Feed substrate and feed residual samples from digestibility measurements were re-ground through a 1-mm screen and ball ground before N and ^{15}N enrichment (only feed substrate) analysis. The N content was determined by flash combustion with gas chromatography and thermal conductivity detection (Carlo Erba Instrumentals, Milan, Italy). The enrichment of ^{15}N in the bacterial samples was analysed by continuous flow measurement of ^{15}N using a combustion analyser interfaced with a stable isotope ratio mass spectrometer (VG Isotech, Middlewich, UK).

Rumen VFA were quantified using GLC (model 5890; Hewlett-Packard, Wilmington, DE) with a capillary column (30 m × 0.32 mm × 1 μm; ZB-FFAP; Phenomenex Inc., Torrance, CA) and flame ionization detection. Crotonic acid was used as internal standard. The oven temperature was maintained at 150°C for 1 min, increased by 5°C/min to 195°C, and held at this temperature for 2.5 min. The injector temperature was 225°C, the detector temperature was 250°C, with helium as the carrier gas. Rumen NH_3 concentration was determined by the

salicylate-nitroprusside-hypochlorite method using a segmented flow analyzer (Rhine *et al*., 1998). Methane and H_2 were measured using a gas chromatograph (model 4900; Varian Inc., Middelburg, the Netherlands) equipped with a 10 m porous polymer column and thermal conductivity detector.

Analisis for total methanogens and bacteria determination were conducted in the same manner as describen in Chapter 2. Protozoa cells were counted using a Neubauer Improved Bright-line chamber as described by Veira *et al*. (1983) and the ciliates were identified according to Dehority (1993).

Calculations and statistical analysis

Gas production volume (total, CH_4 and H_2) was corrected for temperature (0°C) and pressure (101.325 kPa) conditions.

The daily amount of N in SAM (mg/d) was calculated as (Wang *et al*., 2000)

$$N_{SAM} = \frac{{}^{15}N_{FR} \times N_{FR}}{{}^{15}N_{SAM}}$$

where

${}^{15}N_{FR}$ = ${}^{15}N$ enrichment in feed residues (%),

N_{FR} = amount of N in feed residues (mg/d), and

${}^{15}N_{SAM}$ = ${}^{15}N$ enrichment in N of the SAM (%).

The daily amount of N in LAM was calculated in a similar way but using the ${}^{15}N$ enrichment and N concentration in the effluent residues and ${}^{15}N$ enrichment in LAM. Total

microbial N was quantified as the sum of N in LAM and SAM. The efficiency of microbial synthesis (EMPS) was defined as miligram of microbial N per gram of OM disappeared.

The balance of MH was calculated as MH produced (MHP) during fermentation plus total MH recovered (MHR) in the form of VFA (MHR_{VFA}), CH_4 (MHR_{CH4}) and H_2 (MHR_{H2}). The VFA produced per day were calculated based on VFA molar concentration in the fermenters and effluent volume. The amount of MHP and MHR_{VFA} were estimated from the daily amount of acetate (C2), propionate (C3), butyrate (C4), isovalerate (Ci5) and valerate (C5) (Mitsumori *et al*., 2012). The amount of MHR_{CH4} was calculated based on CH_4 production (mmol), considering that 4 moles of H_2 are needed to produce a mole of CH_4. In the case of MHR_{H2}, it was equivalent to H_2 production. Calculations were made using the following equations:

MHP (mmol/d) = (2 × C2) + C3 + (4 × C4) + (2 × Ci5) + (2 × C5);

MHR (mmol/d) = MHR_{VFA} + MHR_{CH4} + MHR_{H2};

MHR_{VFA} (mmol/d) = (2 × C3) + (2 × C4) + C5;

MHR_{CH4} (mmol/d) = CH_4 × 4;

MHR_{H2} (mmol/d) = H_2;

Recovery = MHR / MHP.

Data were analyzed using a mixed model procedure (SAS Inst. Inc., Cary, NC). For all variables, vessel was considered the experimental unit. For feed disappearance, gas production, MH balance, and fermentation variables, the model included the fixed effects of treatment, day and their interactions. For MPS and rumen microbial variables the model included the fixed effect of treatment. Apparatus was considered a random effect. Day was considered a repeated measure in the model where applicable. The variance components were estimated using the

REML method and degrees of freedom were adjusted using the Kenward-Roger option. The Tukey adjustment was used to separate means. The covariance structure was selected based on the lowest Akaike and Bayesian information criteria values. Additionally, linear and quadratic effects of treatments were tested using orthogonal contrasts. Treatment differences and trends were declared significant at $P \leq 0.05$ and $P \leq 0.10$, respectively.

4.3. Results

There was no effect of NOP on DMD and OMD ($P > 0.50$; Table 4.2). Total gas production averaged 1.12 L per day and was not affected by treatments ($P = 0.71$).However, CH_4 production was linearly decreased (< 0.01) by 76.0, 84.5 and 85.6% with inclusion of 5, 10 and 20 mg of NOP, respectively. Methane was also decreased ($P < 0.01$) by about 86.2% when expressed as mL g^{-1} DM or mL g^{-1} DMD with the addition of 20 mg of NOP. Hydrogen gas production increased linearly (up to 216.9%) with NOP supplementation ($P < 0.01$).

Fermentation liquid pH tended to linearly ($P = 0.07$) and quadratically (P=0.08) increase with increasing level of NOP (Table 4.3). Total VFA concentration was not affected ($P = 0.99$), but molar proportion of acetate was decreased with addition of 20 mg of NOP ($P < 0.01$) and isovalerate linearly increased with different levels of NOP ($P < 0.01$). A tendency for a linear increase in butyrate ($P = 0.09$) and a tendency for a quadratic effect for valerate ($P = 0.07$) were also observed with NOP. There was no effect of treatment on molar proportion of propionate, isobutyrate, caproate or the A:P ratio ($P > 0.24$). The sum of propionate, butyrate and valerate (P+B+V) was linearly increased with increasing levels of NOP ($P = 0.01$). A tendency for a quadratic effect ($P = 0.06$) was observed with NOP addition for NH_3 concentration.

Table 4.2. Effect of inclusion level of 3-nitrooxypropanol on digestibility and gas production

	Treatment[1]					*P*-value[2]		
Item[3]	0	5	10	20	SEM	Trt	Lin	Quad
DMD, g/kg DM	569	560	561	562	7.3	0.79	0.63	0.44
OMD, g/kg OM	569	566	577	568	8.0	0.50	0.60	0.90
Gas production								
Total, L/d	1.12	1.07	1.14	1.14	0.05	0.71	0.56	0.88
CH_4, mL/d	27.8[a]	6.7[b]	4.3[b]	4.0[b]	0.95	<0.01	<0.01	<0.01
CH_4, mL/g DM	2.82[a]	0.7[b]	0.44[b]	0.39[b]	0.17	<0.01	<0.01	<0.01
CH_4, mL/g DMD	4.93[a]	1.25[b]	0.78[b]	0.69[b]	0.29	<0.01	<0.01	<0.01
H_2, mL/d	13.1[b]	33.9[a]	40.0[a]	41.6[a]	3.91	<0.01	<0.01	<0.01

[1]Treatments are expressed as mg of 3-nitrooxypropanol.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[3]DM = Dry matter; DMD = Dry matter disappearance; OM = Organic matter; OMD; Organic matter disappearance.

[a, b] Within a row, means without a common superscript differ ($P < 0.05$).

Table 4.3. Effect of inclusion level of 3-nitrooxypropanol on rumen fermentation variables

Variable	Treatment[1]					*P*-value[2]		
	0	5	10	20	SEM	Trt	Lin	Quad
pH	6.96	6.98	7.00	6.99	0.01	0.10	0.07	0.08
Total VFA, m*M*	33.9	33.7	33.6	33.7	1.69	0.99	0.96	0.91
VFA, mol/100 mol								
Acetate (A)	36.8[a]	35.2[ab]	35.3[ab]	33.6[b]	0.65	<0.01	<0.01	0.71
Propionate (P)	20.4	20.8	21.4	20.5	1.03	0.92	0.95	0.52
Butyrate (B)	21.1	21.5	21.8	22.9	0.78	0.37	0.09	0.82
Valerate (V)	15.9	15.7	15.1	16.0	0.41	0.32	0.82	0.07
Isobutyrate	0.45	0.47	0.48	0.47	0.02	0.63	0.35	0.36
Isovalerate	1.59[b]	2.35[a]	2.07[a]	2.29[a]	0.13	<0.01	<0.01	0.03
Caproate	3.70	4.17	4.06	4.23	0.32	0.53	0.27	0.55
P+B+V	57.4	57.8	58.1	59.4	0.70	0.09	0.01	0.07
A:P ratio	1.84	1.73	1.66	1.68	0.10	0.51	0.24	0.35
NH_3, m*M*	6.59	6.13	6.18	6.31	0.19	0.16	0.40	0.06

[1]Treatments are expressed as mg of 3-nitrooxypropanol.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[a, b] Within a row, means without a common superscript differ ($P < 0.05$).

Metabolic hydrogen produced or recovered was not affected by NOP ($P > 0.53$; Table 4.4). Metabolic hydrogen recovered as VFA was not affected ($P = 0.82$); however, MHR_{CH4} was decreased (up to 85.9%) and MHR_{H2} was increased ($P < 0.01$; up to 208.3%) with NOP in both a linear and quadratic manner ($P < 0.01$). When MHR was expressed as a molar proportion, MHR_{VFA} was increased (linear and quadratic effects, $P < 0.01$) by up to 13.9% with NOP. The molar proportion of MH_{CH4} was decreased with different levels of NOP (linear and quadratic effects, $P < 0.01$), with the addition of 10 and 20 mg of NOP showing the greatest effects (81.9 and 84.2% reduction, respectively). Addition of NOP to the diet increased ($P < 0.01$) the molar proportion of MHR_{H2}, but only with 10 and 20 mg of NOP (linear and quadratic effects, $P < 0.01$). As a result, total MH recovery, expressed as a proportion of that produced, was decreased with NOP (linear and quadratic effects, $P < 0.01$).

The total copy number of 16S rRNA gene for bacteria and methanogens in the liquid phase, and total protozoa cells were not affected by treatment ($P > 0.12$; Table 4.5); however, there was a tendency for a linear reduction ($P = 0.06$) in the total copy number of bacterial 16S rRNA gene and a reduction ($P < 0.01$) of that for methanogens present in the solid phase ($P < 0.01$). Total bacterial N, SAM, and EMPS were not affected by NOP ($P > 0.19$). However a linear reduction for LAM ($P = 0.04$) with increasing levels of NOP was observed.

Table 4.4. Effect of inclusion level of 3-nitrooxypropanol on metabolic hydrogen balance

Item	Treatment				SEM	*P* - value[1]		
	0	5	10	20		Trt	Lin	Quad
Produced, mmol/d	42.1	43.2	42.3	43.4	2.63	0.96	0.74	0.99
Recovered, mmol/d	25.0	23.1	22.5	23.2	1.57	0.53	0.40	0.27
VFA	19.4	20.4	19.9	20.7	1.33	0.82	0.47	0.91
CH_4	5.0^{a}	1.2^{b}	0.8^{b}	0.7^{b}	0.19	<0.01	<0.01	<0.01
H_2	0.6^{b}	1.5^{a}	1.6^{a}	1.9^{a}	0.22	<0.01	<0.01	0.01
Recovered, mol/100 mol								
VFA	78.2^{b}	88.3^{a}	89.1^{a}	88.2^{a}	0.87	<0.01	<0.01	<0.01
CH_4	19.5^{a}	5.5^{b}	3.5^{c}	3.1^{c}	0.49	<0.01	<0.01	<0.01
H_2	2.1^{b}	6.4^{ab}	7.4^{a}	8.6^{a}	0.52	<0.01	<0.01	<0.01
Recovery[3]	0.59^{a}	0.53^{b}	0.53^{b}	0.54^{b}	0.01	<0.01	<0.01	<0.01

[1]Treatments are expressed as mg of 3-nitrooxypropanol.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[3]Metabolic hydrogen recovered as a proportion of that produced.

[a, b] Within a row, means without a common superscript differ ($P < 0.05$).

Table 4.5. Effect of inclusion level of 3-nitrooxypropanol on rumen microbes and microbial protein synthesis

Item	Treatment[1]				SEM	*P* - value[2]		
	0	5	10	20		Trt	Lin	Quad
Rumen microbes								
Total protozoa, × 10^3 cells/mL[3]	4.20	5.60	5.70	5.90	1.82	0.61	0.87	0.39
Liquid phase								
Bacteria, × 10^9 copies/mL	3.12	3.39	6.08	8.24	2.07	0.55	0.19	0.90
Methanogens, × 10^6 copies/mL	8.09	3.95	5.08	5.25	1.88	0.12	0.99	0.98
Solid phase								
Bacteria, × 10^{10} copies/g	14.5	16.5	9.5	9.4	3.22	0.21	0.06	0.82
Methanogens, × 10^6 copies/g	5.25[a]	0.50[b]	0.18[b]	0.17[b]	0.48b	<0.01	<0.01	<0.01
Bacterial N, mg/d								
Total	169	136	145	133	15.5	0.38	0.19	0.47
LAM[4]	33.2	34.6	33.6	26.2	2.5	0.11	0.04	0.18
SAM[4]	136	102	111	107	14.3	0.37	0.29	0.31
EMPS, mg bacterial N/g OM disappeared[4]	32.2	26.7	28.7	25.6	3.00	0.45	0.21	0.66

[1]Treatments are expressed as mg of 3-nitrooxypropanol.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[3]Samples for protozoa counting were obtained at the time of feed bag exchange by squeezing the bags containing feed residuals after 24 and 48 h incubation to expel excess liquid.

[4]LAM = Liquid associated microorganisms; SAM = Solid associated microorganisms; EMPS = Efficiency of microbial protein synthesis.

[a, b] Within a row, means without a common superscript differ ($P < 0.05$).

4.4. Discussion

This study is the first to evaluate the effects of different inclusion levels of NOP on CH_4 production, rumen fermentation and microbial community using the Rusitec system.

In previous studies, the use of NOP did not cause any negative effects on nutrient digestibility when it was evaluated in beef (Romero-Perez, *et al*., 2014) or dairy (Reynolds, *et al*., 2014) cattle. However, the largest dose used in each of those experiments was equivalent to 0.25 and 0.12 mg NOP/g DM for the beef and dairy experiments, respectively. The doses of NOP (5, 10 and 20 mg/d) used in the present study were equivalent to 0.5, 1.0 and 2.0 mg NOP/g DM and these levels were expected to provide insight into possible negative effects of substantial CH_4 reduction on feed digestion. Despite the relatively large doses of NOP and the substantial inhibition of CH_4 production, no negative effects on DMD or OMD occurred. Likewise, CH_4 inhibitors similar to NOP such as BES, which is a structural analog of methyl-CoM, have been reported to reduce CH_4 emissions by up to 52% *in vitro* with no effect on DMD (Dong *et al*., 1999; O'Brien *et al*., 2014). Indeed, other inhibitors such as BCM have been reported to reduce CH_4 emissions in goats (Mitsumori *et al*., 2012) by more than 90% with no effect on DM digestibility in the total tract. When BCM was dosed to sheep at 4.5 mg/kg BW (Sawyer *et al*., 1974), DM digestibility in the total tract was increased by 12% even though CH_4 production was decreased by 85%.

The observed tendency for a linear increase in pH of the fermenter liquid with NOP addition is consistent with previous *in vivo* studies. While the increase in pH in Rusitec was only 0.04 units, NOP has been reported to increase minimum rumen pH by 0.23 units in beef (Romero-Perez *et al*., 2014, 2015) and 0.16 units in dairy cattle, as well as reduce the time that pH remains below 6 (Reynolds *et al*., 2014). This difference between *in vivo* and *in vitro* is likely

to be due to the strong buffering capacity of the McDougall saliva used in the Rusitec system.

The observed VFA concentrations were low but not unexpected based on previous Rusitec experiments, which have reported similar values (Fraser *et al*., 2007; Nanon *et al*., 2014). 3-Nitrooxypropanol did not affect the concentration of total VFA, which is in agreement with the lack of effect on DMD, OMD, and total gas production. Increasing doses of NOP have been reported to linearly reduce total VFA concentration in the rumen of beef cattle (Romero-Perez *et al*., 2014; greatest dose used: 217 mg NOP/kg DMI). However, in a subsequent experiment by Romero-Perez *et al*. (2015) using a greater dose (280 mg NOP/kg DMI) total VFA concentration was not affected. Reynolds *et al*. (2014) and Haisan *et al*. (2014) used lower doses in dairy cows (up to 124 mg NOP/kg DMI and 130 mg NOP/kg DMI, respectively); but, only Reynolds *et al*. (2014) reported a reduction in total VFA. Together these studies indicate that NOP has no clear effect on concentration of total VFA in the rumen.

Typically, the use of NOP (Haisan *et al*., 2014; Martínez-Fernández., 2014; Romero-Perez *et al*., 2014, 2015) and other CH_4 inhibitors like BCM (McCrabb *et al*., 1997), BES (Lee *et al*., 2009; O'Brien *et al*., 2014) and chloroform (Knight *et al*., 2011) has decreased the rumen molar proportion of acetate and increased the molar proportion of propionate with concomitant reduction in the acetate to propionate ratio. When CH_4 production is inhibited, an increase in propionate production is expected because after CH_4, propionate is considered the most important MH sink in the rumen (McAllister and Newbold, 2008). In the present study, the acetate molar proportion was decreased, whereas propionate was not affected. When Reynolds *et al*. (2014) administered 2.5 g/d of NOP to dairy cows, the rumen concentration of propionate was not affected, and only a 2% increase was observed when propionate was expressed as a molar proportion of total VFA. However, in that experiment the CH_4 reduction observed (6.7%) was

not as large as in the present study. Recently, de Matos *et al*. (2015) observed a 34% reduction in CH_4 production from dairy cows consuming NOP without an effect on propionate concentration. It is possible that the lack of effect of NOP on propionate molar proportion was because MH was redirected to non-determined reduced end products instead (Immig *et al*., 1996). Ungerfeld (2015) pointed out that some propionate producers may not adapt well to continuous cultures and do not benefit from favorable thermodynamic conditions for propionate production occurring when methanogenesis is inhibited. The tendency for linear increase in the molar proportion of butyrate observed herein has been previously observed *in vivo* with NOP addition (Reynolds *et al*., 2014; Romero-Perez *et al*., 2014, 2015).

Butyrate is a reduced end product of fermentation whose formation from pyruvate requires H_2 from NADH, therefore butyrate is also considered a H_2 sink (Immig, 1996). This is of special interest because by shifting H_2 disposal from CH_4 to propionate or butyrate, more energy is available to the animal (Van Nevel and Demeyer, 1996).

An unusually high molar proportion of valerate was observed for all treatments. Although a redirection of MH to products like valerate is expected when methanogenesis is inhibited, valerate was also elevated for the control treatment. Previous Rusitec experiments have also reported high valerate concentrations for control treatments (Li *et al*., 2012). Although continuous culture systems are known to generate relatively high valerate concentrations, no clear explanation for this has been proposed.

Methane production observed in the present experiment for the control treatment is in accordance with previous experiments in our lab (Li *et al*., 2012; Meale *et al*., 2014; Nanon *et al*., 2014). The lack of effect of NOP on total gas production and the reduction in CH_4 production is in agreement with a previous *in vitro* batch culture experiment in which 33 and 66 m*M* of NOP

(equivalent to 8 and 16 mg NOP/g DM, respectively) did not affect total gas production but decreased CH_4 emissions by 86 and 95%, respectively, when these levels were added to a diet of 60% alfalfa hay and 40% oat grain (Martínez-Fernández *et al*., 2014). The greater CH_4 reduction of 86 and 95% observed by Martínez-Fernández *et al*. (2014) may be attributed to the larger NOP doses evaluated in their study compared to the doses used herein. However, doubling the NOP dose from 5 to 10 mg/d and from 10 to 20 mg/d in the present study did not reduce CH_4 production in the same manner. The 75.5, 84.4 and 86.2% reduction in CH_4 with 5, 10 and 20 mg NOP/d, respectively, may suggest a point along a curve of increasing NOP dose where there may not be further significant CH_4 reduction with an additional increment. Because there is a possibility that methanogen species are affected differently by NOP, as discussed below, a complete inhibition of rumen methanogenesis with NOP addition was not expected. Overall, in agreement with the present study, the use of NOP has consistently decreased CH_4 emissions when evaluated in sheep (Martínez-Fernández *et al*., 2014), beef (Romero-Perez *et al*., 2014, 2015), and dairy cattle (Haisan *et al*., 2014; Reynolds *et al*., 2014) and results obtained in this study are in agreement with these findings.

An elevated H_2 concentration in the gas mixture (1.18 mL/100 mL) was observed for the control. Hydrogen used for CH_4 production in the rumen occurs in three key states: H_2, reduced cofactors (e. g. NADH and NADPH), and free protons (H^+) (Hegarty and Gerdes, 1999). Hydrogen gas in the rumen head space rarely accumulates to concentrations greater than 1 mL/100 mL; but, concentrations over this value can also be expected (Hegarty and Gerdes, 1999). The H_2 partial pressure in a system such as Rusitec, where gas is collected in a bag that expands may differ from other systems. Concentration of H_2 can range from non-detectable levels to 5 mL/100 mL, in continuous cultures, up to 38 mL/100 mL, in batch cultures, including

fermentations where methanogenesis was inhibited (Ungerfeld, 2015). Because changes in the partial pressure of H_2 may have an impact on the concentration of H_2 dissolved in the aqueous phase (which together with H^+ concentration determines the redox potential in the rumen), the extent of oxidation of feedstuffs may also be affected (Hegarty and Gerdes, 1999).

The use of specific CH_4 inhibitors has been related to H_2 accumulation both *in vitro* (Immig *et al*., 1996; Soliva *et al*., 2011; O'Brien *et al*., 2014) and *in vivo* using direct measurement (Immig *et al*., 1996; Mitsumori *et al*., 2012), or stoichiometric calculation (Mitsumori *et al*., 2012; Romero-Perez *et al*., 2015). The accumulation of H_2 observed in the present experiment indicates a redirection of MH which is in agreement with previous observations using CH_4 inhibitors (Immig *et al*., 1996; Soliva *et al*., 2011; Mitsumori *et al*., 2012; O'Brien *et al*., 2014). Hydrogen is a central metabolite in rumen fermentation and its partial pressure is an important determinant of rumen methanogenesis (Hegarty and Gerdes 1999). Because H_2 is usually thought to act as a feedback inhibitor in the fermentation process it has to be removed from the rumen (Immig *et al*., 1996). Otherwise, cofactors such as NADH, NADPH and reduced ferridoxins that are necessary for continuous glycolytic activity by the rumen microbial consortia cannot be regenerated with consequent reduction in fermentation rate, feed intake and digestibility (Leng, 2014). Under normal rumen conditions, the formation of CH_4 within biofilms in the rumen prevents a rise in the partial pressure of H_2 (Leng, 2014). However, the present results indicate that CH_4 inhibitors like NOP can drastically reduce CH_4 emissions and increase H_2 without affecting digestibility. The reduction in CH_4 emissions and the increase in H_2 without affecting digestibility may be explained by the work of Leng (2014), who indicated that methanogenesis in biofilms is dependent on interspecies transfer of electrons via H_2 or formate. When CH_4 inhibitors are used, formate rather than H_2 may be produced within the

biofilms. This shift from H_2 to formate would keep H_2 partial pressure low within the biofilm allowing the regeneration of cofactors and fermentation to continue. Formate would eventually diffuse to the liquid phase to form HCO_3^- and H_2, with the latter escaping from the rumen through breath (Leng, 2014).

In the present study the MH recovery observed for the control treatment was low but not unexpected because similar values have been previously reported (Machmüller *et al*., 1998). The use of CH_4 inhibitors has been reported to reduce MH recovery rates (Van Nevel and Demeyer, 1981; Immig *et al*., 1996; Goel *et al*., 2009; Zhang *et al*., 2012). Although MHP and MHR were not affected by NOP in the present study, MH recovery (as a proportion of that produced) decreased by up to 10.7% with NOP, mainly because the reduction in MHR_{CH4} was not compensated for by increased MHR_{H2}. The reduction in MH recovery percentage might be indicative of accumulation of non-determined reduced end products like formate (Immig *et al*., 1996), because formate can accumulate with the use of CH_4 inhibitors (Leng, 2014). However, formate was not quantified in this experiment. The increase in molar proportion of MHR_{VFA} is in agreement with the increase in the molar proportion of the sum of propionate, butyrate and valerate. This is because MHR_{VFA} was calculated based on concentrations of these same VFA's. As expected from the reduction in CH_4 and concomitant increase in H_2 production, the molar proportion of MHR_{CH4} was decreased and that for MHR_{H2} was increased by NOP. However, most of MH that was not used for CH_4 production was redirected to VFA. This assertion is exemplified by the results that, when 20 mg of NOP was supplied, the molar proportion of MHR_{CH4} was decreased by 16.4% units (i.e., from 19.5% for control to 3.1% with NOP), that for MHR_{VFA} was increased by 10.0% units (i.e., from 78.2% for control to 88.2% with NOP) and MHR_{H2} was increased by 6.5% units (i.e., from 2.1% for control to 8.6% for NOP). The

redirection of MH to useful products like VFA could represent an energetic benefit for ruminants when NOP is supplied. Recently, Ungerfeld (2015) carried out a meta-analysis to quantify the shifts in MH sinks when methanogenesis was decreased *in vitro*, and concluded that reducing methanogenesis increases MH incorporation into propionate in batch cultures, but not in continuous cultures and that butyrate was not affected in either system, although there were interactions with experiment. However, the specific use of chemicals with direct inhibitory effect on methanogenesis increased MH incorporation into both propionate and butyrate. In accordance with our findings, Ungerfeld (2015) observed that decreased methanogenesis was related to increased MH incorporation into H_2 and decreased MH recovery rate, and suggested that major MH sinks such as formate, microbial biomass, and reductive acetogenesis were unaccounted for.

The addition of NOP in the present study did not affect total copy number of 16S rRNA gene for methanogens in the liquid phase. The use of NOP has been reported to reduce CH_4 emissions without affecting methanogen abundance, providing evidence that NOP may alter the function of specific microorganisms rather than the microbial community itself (Romero-Perez *et al*., 2014). Methanogens that are able to synthesize CoM intracellularlly (*Methanobacterium mobile*; *Methanobrevibacter smithii*), showed low levels of CoM uptake (< 10%) as compared to methanogens that cannot synthesize it (*Methanobrevibacter ruminantium* M1) and are therefore likely to be more resistant to BES (Balch and Wolfe., 1979; Ungerfeld., *et al*., 2004). When BES was evaluated using dual-flow continuous culture fermenters, a partial selection for non-sensitive methanogens like *Methanobrevibacter smithii* was observed (Karnati *et al*., 2008). NOP has a similar structure to CoM and BES, and thus, it is possible that methanogens that synthesize CoM would be less sensitive to NOP. Therefore, NOP would modify the methanogen community by decreasing sensitive methanogens and proportionally increasing non-sensitive species. Location

within the rumen (liquid phase, solid phases and epithelium) from which methanogens are detected plays a role in the type of methanogens identified (Hook *et al*., 2010). Thus, the effect of NOP on methanogen community may vary depending on the type of sample analysed. In the present experiment, the total copy number of methanogenic 16S rRNA gene in the solid phase was reduced by 90.5, 96.6 and 96.7% with the addition of 5, 10 and 20 mg of NOP, which corresponds to the 75.2, 84.4 and 86.2% reductions in CH_4 emissions, respectively. The use of NOP may drastically reduce CH_4 only when total methanogens are decreased.

The overall process of methanogenesis in the rumen allows formation of ATP which is used by methanogens for maintenance and growth (Van Nevel and Demeyer, 1995), thus the inhibition of methanogenesis by NOP might de-energize rumen methanogens and decrease growth rate with consequent reduction in the methanogen population. Methanogens not inhibited by NOP would have more H_2 available and might generate more ATP per mol of CH_4 produced (Keltjens and Vogels 1996). From previous *in vivo* experiments (Romero-Perez *et al*., 2015) we know that although CH_4 production and methanogens are dramatically decreased with long-term addition of NOP, recovery takes place relatively fast as CH_4 emissions are restored to control levels within days of NOP no longer being added.

Protozoa and methanogens have a symbiotic relationship where protozoa produce H_2, serve as hosts for methanogens and also protect them from oxygen toxicity. In contrast, methanogens remove H_2 allowing the fermentation of OM to proceed resulting in more efficient ATP production by the protozoan host (Morgavi *et al*., 2010). Thus, it is expected that changes in the protozoa community may affect methanogens and vice versa. In the present experiment no effect was observed on total protozoa or total copy number of 16S rRNA genes for methanogens in the liquid phase with NOP. Previous experiments evaluating NOP showed no effect on total

copy number of protozoa or bacteria (Haisan *et al*., 2014; Martínez-Fernández *et al*., 2014; Romero-Perez *et al*., 2014). When BES was evaluated *in vitro*, the generic distribution of protozoa including *Entodinium*, *Diplodiniinae* (*Diplodinium*, *Eudiplodinium*, *Ostracodinium*, *Metadinium*, *Enoploplastron*, and *Polyplastron*), *Epidinium*, *Isotricha* and *Dasytricha*, was not affected (Karnati *et al*., 2008). Moreover, an increase in total copy number of 18S rRNA gene for protozoa was reported when adding 2 g of NOP to a beef cattle diet; but, the mechanisms behind this increase were unclear (Romero-Perez *et al*., 2015). Although the total copy number of 16S rRNA gene for bacteria was not affected in previous studies evaluating NOP (Haisan *et al*., 2014; Martínez-Fernández *et al*., 2014; Romero-Perez *et al*., 2014, 2015), it is likely that changes observed in the present experiment for rumen fermentation variables including increased pH, decreased acetate, increased P+B+V and increased valerate, were caused by the tendency for linear reduction in total bacteria and possibly by a rearrangement of the bacterial community structure (Zhou et al., 2015), either by a direct or more probably by indirect effects of NOP including the accumulation of H_2 or formate.

The increase in isovalerate observed in the present study was also observed previously in beef (Romero-Perez *et al*., 2014, 2015). Isovalerate is produced by the deamination and decarboxylation of branched chain amino acids (Andries *et al*., 1987), and thus, it was expected that an increase in the molar proportion of isovalerate with NOP supplementation would increase NH_3 concentration. However, a tendency for quadratic decrease in NH_3 was observed. Increased isovalerate with no effect on NH_3 can occur if EMPS increases (Goel *et al*., 2009); however, in the present experiment EMPS was not affected. Previous experiments using CH_4 inhibitors have shown no effect (Goel *et al*., 2009) or even increases in the EMPS. However, it has been suggested that CH_4 inhibition might decrease EMPS because CH_4 acts as a MH sink in the rumen

that affects the formation of certain end products and increases the amount of ATP generated (Van Nevel and Demeyer, 1981).

The addition of NOP to a mixed diet decreased CH_4 (mL g^{-1} DM) production up to 86.2% with no effects on digestibility. The 75.5, 84.4 and 86.2% reduction in CH_4 with 5, 10 and 20 mg NOP/d respectively, may suggest a point along a curve of increasing NOP dose where there may not be further significant CH_4 reduction with an additional increment. Fermentation was modified with NOP by decreasing the molar proportion of acetate, increasing isovalerate, and the sum of propionate, butyrate and valerate. The decrease in CH_4 production indicated a redirection of MH mainly to VFA, which could be beneficial for animal production. However, MH was also utilized for H_2 production which represents a loss of feed energy. The reduction in the total copy number of 16S rRNA gene for methanogens in the solid phase was in agreement with CH_4 reduction but the lack of effect on copy number of methanogens in the liquid phase suggests that the species in this phase may be less sensitive to NOP.

4.5. Literature cited

Andries, J. I., Buysse, F. X., De Brabander, D. L., and Cottyn, B.G. 1987. Isoacids in ruminant nutrition: their role in ruminal and intermediary metabolism and possible influences on performances - A review. Anim. Feed Sci. Technol. 18:169–180.

Association of Official Analytical Chemists (AOAC). 2006. Official Methods of Analysis, 18th ed., revisions 1. AOAC International, Gaithersburg, MD, USA.

Balch, W. E., and Wolfe, R. S. 1979. Transport of Coenzyme M (2-Mercaptoethanesulfonic acid) in *Methanobacterium ruminantium*. J. Bacteriol. 137:264–273.

Canadian Council on Animal Care, 2009. CCAC guidelines on: The care and use of farm animals in research, teaching and testing. Ottawa, ON, Canada. http://www.ccac.

ca/Documents/Standards/Guidelines/Farm_Animals.pdf. (Accessed April 13, 2015.)

Chen, Y., Penner, G. B., Li, M., Oba, M., and Guan, L. L. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. Appl. Environ. Microbiol. 77:5770–5781.

Chen, Y., Oba, M., and Guan, L., 2012. Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. Vet. Microbiol. 159:451–459.

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

Dehority, B. A.. 1993. Laboratory manual for classification and morphology of ruminal ciliate protozoa. CRC Press, Boca Raton, FL.

de Matos, L. F., Harper, M. T., Lopes, J., Giallongo, F., Oh, J., Gruen, D., Hristov, A. N., Kindermann, M., and Duval, S. 2015. Effect of 3-nitrooxypropanol on ruminal fermentation, methane and hydrogen emissions, and methane isotopic composition in dairy cows. J. Anim. Sci. 93(s3):246.

Dong, Y., Bae, H. D., McAllister, T. A., Mathison, G. W., and Cheng, K.-J. 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.

Duval, S., and Kindermann, M. 2012. Use of nitrooxy organic molecules in feed for reducing enteric methane emissions in ruminants, and/or to improve ruminant performance, International Patent Application WO 2012/084629 A1, World Intellectual Property Organization. Geneve, Switzerland.

Fraser, G. R., Chaves, A. V., Wang, Y., McAllister, T. A., Beauchemin, K. A., and Benchaar, C. 2007. Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems. J. Dairy Sci. 90:2315–2328.

Goel, G., Makkar, H. P. S., Becker, K. 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. Br. J. Nutr. 101:1484–1492.

Haisan J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, and D. R., Oba, M. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

Hegarty, R. S., and Gerdes, R. 1999. Hydrogen production and transfer in the rumen. Rec. Adv. Anim. Nutr. Austr. 12:37–44.

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

Immig, I., Demeyer, D., Fiedler, D., Van Nevel, C., and Mbanzamihigo, L. 1996. Attempts to induce reductive acetogenesis into a sheep rumen. Archiv. für Tierernaehrung. 49(4):363-370.

IPCC. 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels,Y.

Xia, V. Bex, P. M. Midgley, editors]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

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

Karnati, S. K. R., Yu, Z., Firkins, J. L. 2008. Investigating unsaturated fat, monensin, or bromoethanesulfonate in continuous cultures retaining ruminal protozoa. II. Interaction of treatment and presence of protozoa on prokaryotic communities. J. Dairy Sci. 92:3861–3873.

Keltjens, J. T. and Vogels, G. D. 1996. Metabolic regulation in methanogenic Archaea during growth on hydrogen and CO_2. Environ. Monit. Assess. 42:19–37.

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P., Tricarico, J. M. 2013. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97:1–31.

Knight, T., Ronimus, R. S., Dey, D., Tootill, C., Naylor, G., Evans, P., Molano, G., Smith, A., Tavendale, M., Pinares-Patiño, C. S., and Clark, H. 2011. Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. Anim. Feed. Sci. Technol. 166–167:101–112.

Lee, S. Y., Yang, S. H., Lee, W. S., Kim, H. S., Shin, D. E., and Ha, J. K., 2009. Effect of 2-bromoethanesulfonic acid on *in vitro* fermentation characteristics and methanogen population. Asian-Aust. J. Anim. Sci. 22:42–48.

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54:519-543.

Li, Y., He, M., Li, C., Forster, R., Beauchemin, K. A., and Yang, W. 2012. Effects of wheat dried distillers' grains with solubles and cinnamaldehyde on *in vitro* fermentation and protein degradation using the Rusitec technique. Arch. Anim. Nutr. 66:131–148.

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

McCrabb, G. J., Berger, K. T., Magner, T., May, C., and Hunter, R. A. 1997. Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. Aust. J. Agric. Res.48:323–329.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43:99–109.

Machmüller, A., Ossowski, D. A., Wanner, M., and Kreuzer, M. 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Anim. Feed Sci. Tech. 71:117–130.

Martínez, M. E., Ranilla, M. J., Ramos, S., Tejido, M. L., and Carro, M. D. 2009. Effects of dilution rate and retention time of concentrate on efficiency of microbial growth, methane production, and ruminal fermentation in Rusitec fermenters. J. Dairy Sci. 92:3930–3938.

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

Meale, S. J., Ding, S., He, M. L., Dugan, M. E. R., Ribeiro Jr., G. O., Alazzeh, A. Y., Holo, H., Harstad, O. M., McAllister, T. A., and Chaves, A.V. 2014. Effect of Propionibacterium freudenreichii on ruminal fermentation patterns, methane production and lipid

biohydrogenation of beef finishing diets containing flaxseed oil in a rumen simulation technique. Can. J. Anim. Sci. 94:685–695.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., and McSweeney, C. S. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108:482–491.

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

Nanon, A., Suksombat, W., Beauchemin, K. A., and Yang, W. Z. 2014. Assessment of lemongrass oil supplementation in a dairy diet on *in vitro* ruminal fermentation characteristics using the rumen simulation technique. Can. J. Anim. Sci. 94:731–736.

Nutt, B. G., Holloway, J. W., and Butts, W. T. 1980. Relationship of rumen capacity of mature angus cows to body measurements, animal performance and forage consumption on pasture. J Anim. Sci. 51:1168–1176.

O'Brien, M., Navarro-Villa, A., Purcell, P. J., Boland, T. M., and O'Kiely, P. 2014. Reducing *in vitro* rumen methanogenesis for two contrasting diets using a series of inclusion rates of different additives. Anim. Product. Sci. 54:141–157.

Ogimoto, K., and Imai, S. 1981. Atlas of Rumen Microbiology. Japan Scientific Societies Press, Tokyo.

Prakash, D. 2014. Methyl-coenzyme M reductase: Elucidating the process of activation and study of the effect of the methanogenesis inhibitor 3-nitrooxypropanol (Doctoral dissertation). Auburn University. https://etd.auburn.edu/handle/10415/4463.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of incremental rumen doses of 3-nitrooxypropanol on methane production, digestion and rumen parameters in lactating dairy cows. J. Dairy Sci. 97:3777–3789.

Rhine, E. D., Sims, G. K., Mulvaney, R. L., and Pratt, E. J. 1998. Improving the Berthelot reaction for determining ammonium insoil extracts and water. Soil Sci. Soc. Am. J. 62:473–480.

Romero-Perez A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682–4693.

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

Sawyer, M. S., Hoover, W. H., and Sniffen, C. J. 1974. Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. J. Anim. Sci. 38:908–914.

Shima, S., and Thauer, R. K. 2005. Methyl-coenzyme M reductase and the anaerobic oxidation of methane in methanotrophic archaea. Curr. Opin. Microbiol. 8:643–648.

Soliva, C. R., Amelchanka, S. L., Duval, S. M., and Kreuzer, M. 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). Br. J. Nutr. 106:114–122.

Thauer, R. K. 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology. 144:2377–2406.

Ungerfeld, E. M., Rust, S. R., Boone, D. R., and Liu, Y. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. J. Appl. Microbiol. 97:520–526.

Ungerfeld, E. M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6(37):1–17.

Van Nevel, C., and Demeyer, D. 1981. Effect of methane inhibitors on the metabolism of rumen microbes *in vitro*. Archiv für Tierernaehrung, 31(2):141–151.

Van Nevel, C., and Demeyer, D. 1995. Feed additives and other interventions for decreasing methane emissions, In Biotechnology in animal feeds and animal feeding [R. J. Wallace, A. Chesson, editors]. VCH Verlagsgesellschaft mbH., Weinheim, Germany. pp. 329–349.

Van Nevel, C.J., and Demeyer, D.I. 1996. Control of rumen methanogenesis. Environ. Monit. Assess. 42:73–97.

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

Veira, D. M., Ivan, M., and Jui, P. Y. 1983. Rumen ciliate protozoa: effects on digestion in the stomach of sheep. J. Dairy Sci. 66:1015–1022.

Zhang, D. F, and Yang, H. J. 2012. Combination effects of nitrocompounds, pyromellitic diimide, and 2-bromoethanesulfonate on *in vitro* ruminal methane production and fermentation of a grain-rich feed. J. Agric. Food Chem. 60:364–371.

Zhou, M., Hernandez-Sanabria, E., Guan, L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Appl. Environ. Microbiol. 75:6524–6533.

Zhou, M., Chen, Y., Haisan, J., Romero-Perez, A., Beauchemin, K. A., Oba. M., Kindermann, M., Duval, S., and Guan, L. L. 2015. Effects of feeding 3-nitrooxypropanol (NOP) on rumen microbial profiles in lactating dairy cows and beef cattle. Proceedings of the ADSA-ASAS Joint Annual Meeting, July 12-16, 2015, Orlando, Florida, USA.

5. Effects of 3-nitrooxypropanol and monensin on methane production using a forage-based diet in Rusitec fermenters

5.1. Introduction

Numerous options have been discussed as strategies to mitigate CH_4 emissions coming from animal agriculture, including the use of MON and inhibitors (Kumar *et al*., 2014). Until recently, the use of enzymatic inhibitors to reduce CH_4 production by ruminants was considered not feasible because of environmental concerns, toxicity to ruminants, inhibition of rumen fermentation, or the loss of effectiveness over time due to microbial adaptation (Immig *et al*., 1996; Kumar *et al*., 2014; Tomkins *et al*., 2009). However, studies evaluating NOP continue to demonstrate that CH_4 production can be decreased without adverse effects on animal health or productivity on a long term basis (Haisan *et al*., 2014; Histrov *et al*., 2015; Romero-Perez *et al*., 2014, 2015a). Moreover, dairy cows consuming NOP in their diet have more energy available for production purposes such as weight gain as a consequence of the decreased loss of energy as CH_4 (Haisan *et al*., 2014; Histrov *et al*., 2015; Romero-Perez *et al*., 2014, 2015a). 3-Nitrooxypropanol is a compound that inhibits the last step of methanogenesis by quenching the active form of MCR (Prakash, 2014). A previous *in vitro* study evaluating NOP in Rusitec fermenters reported that CH_4 was decreased by up to 86% together with a reduction in the proportion of acetate and total methanogens in the solid phase (Romero-Pérez *et al*., 2015b).

[1]A version of this chapter has been submitted to Br. J. Nutr. Romero-Pérez, A., Okine, E. K., Guan, L. L., Duval, S. M., Kindermann, M., and Beauchemin, K. A., 2015. Effects of 3-nitrooxypropanol and monensin on methane production using a forage-based diet in Rusitec fermenters.

Monensin is an antimicrobial that increases feed efficiency and moderately reduces CH_4 production by increasing the flow of MH to propionate production (Beauchemin *et al*., 2008). Although the use of MON was banned as a feed additive in the European Union (EMEA, 2007) and public pressure to reduce the use of antimicrobials in animal agriculture is increasing, the maximum residue limits for MON in bovine species in the European Union was recently increased (EMEA, 2013) and MON is still widely used in many countries including Canada, United States, Mexico, Australia, and New Zealand (Duffield *et al*., 2008).

Simultaneous implementation of different CH_4 mitigation strategies is expected to occur at the farm level, and thus, it is important to evaluate the effect of such combinations on rumen fermentation. It is also important to verify that the use of two different compounds is not mutually exclusive. Due to the different modes of action of NOP and MON, our hypothesis was that the combination of both would lead to a greater CH_4 reduction as compared to the use of either compound alone. Thus, the objective of the present experiment was to evaluate the effects of NOP, MON or the combination of NOP plus MON on the fermentation of a forage-based diet incubated in Rusitec fermenters.

5.2 Materials and Methods

The experiment was conducted at Agriculture and Agri-Food Canada, Lethbridge Research Centre. Animals used as rumen content donors were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

Experimental design, treatments, and diet

The experiment was arranged as a completely randomized block design with 2 blocks (Rusitec apparatuses) and 4 treatments: Control (no additives), 2 mg of NOP (DSM Nutritional Products, AG, Kaiseraugst, Switzerland), 2 mg of MON (Rumensin Premix Monensin with Microtracer®, 200 g/kg, Elanco, Division Eli Lilly Canada, Inc., Guelph, Canada) and the combination of 2 mg of NOP and 2 mg of MON (NOP + MON). The NOP dose was selected based on results from a previous experiment where different levels of NOP were evaluated in the Rusitec (Romero-Pérez *et al*., 2015b), while the MON dose was chosen based on Rusitec experiments where changes in rumen fermentation and CH_4 production were reported (Wallace *et al*., 1981; Bogaert *et al.,* 1990; Jalč *et al*., 1992; Wischer *et al*., 2013). The duration of the experiment was 18 d, including an adaptation period (8 d) without treatment use, a treatment period (7 d), and a final recovery period (3 d) where treatments were discontinued.

Treatments were applied daily b mixing them with 10 g of diet (DM basis; Table 5.1). The diet was previously dried and ground through a 4-mm screen. Feed substrate was weighed into pre-labelled nylon bags (7 × 12 cm; 51 μm mesh opening; Sefar America Inc., New York, USA), sealed with heat and stored at room temperature until utilized.

Table 5.1. Ingredients and chemical composition of the diet.

Item	g/kg of DM
Ingredient[1]	
Barley silage	600
Barley grain, dry-rolled	350
Barley grain, ground	26.9
Calcium carbonate	13.7
Canola meal	5.00
Salt	1.58
Urea	1.10
Molasses, dried	1.08
Vitamin-mineral premix[2]	0.55
Vitamin E (500,000 IU/kg)	0.04
Flavouring agent	0.03
Chemical composition	
DM[3]	919
OM	923
CP	123
NDF	395
ADF	234

[1]All ingredients except barley silage and dry-rolled barley grain were part of a supplement.

[2]Vitamin-mineral premix contained (g/kg of DM): $CaCO_3$,

350.1; CuSO4, 103.7; ZnSO4, 282.3; ethylene diaminedihydriodide (800 g/kg), 1.5; selenium (10g Se/kg), 50.1; $CoSO_4$, 1.0; $MnSO_4$, 145.4; vitamin A (500,000,000 IU/kg), 17.1; vitamin D (500,000,000 IU/kg), 1.7; and vitamin E (500,000 IU/kg), 47.0.

[3]Expressed as g/kg of feed

Rumen simulation technique

Two Rusitec apparatuses (Czerkawski and Breckenridge, 1977) each equipped with eight 1-L volume anaerobic fermenters were used. Each fermenter was outfitted with a site for artificial saliva input and effluent output. Rumen fluid and solid rumen content used as inoculum was obtained 2 h after the morning feeding from 2 rumen fistulated beef cattle fed the same diet as that fed to the Rusitec (Table 5.1). Rumen fluid from two different animals was mixed together and was used as a whole. Solid rumen contents were treated in the same way as liquid. On the first day of the experiment, each fermenter was filled to overflow with a mixture containing 20% of artificial saliva and 80% of strained rumen fluid. Two pre-labelled nylon bags, one containing 10 g of solid rumen digesta, and the other containing 10 g (DM) of diet were also allocated into each fermenter. After 24 h, the nylon bag containing solid rumen digesta was removed and replaced by a nylon bag containing 10 g of diet substrate. Thereafter, one bag was replaced daily so that each bag remained in the fermenter for 48 h except for the last day of the experiment, when one bag in each vessel was removed after 24 h. Collectively, the fermenters were immersed in a water bath maintained at a constant 39ºC and vertically agitated at 0.9 cycles/min. Artificial saliva (McDougall, 1948) modified to contain $(NH_4)_2SO_4$ (Martínez

et al., 2009) was prepared daily and administered at a renewal rate of 2.9% h^{-1} using a peristaltic pump (Model III; Pulse Instrumentation Ltd.; Saskatoon, SA, Canada).

Effluent was collected in 1-L Erlenmyer flasks which in turn were connected to 2-L plastic bags for gas collection (Curity®; Conviden Ltd, Mansfield, MA, USA). At the time of the daily feed bag exchange, rumen fluid pH, total gas production and effluent volume from each fermenter were measured. Starting at d 8, the treatments were administered by mixing them with the feed substrate contained in the nylon bags. During feed bag exchange, the fermenters were flushed with O_2-free CO_2 to maintain an anaerobic environment.

All materials and methods for determining dry matter and nutrient dissappearance, fermentation gases, products of fermentation such as VFA, and NH_3, microbial community, and MPS were conducted as indicated in Chapter 4.

Calculations and statistical analysis

Total gas volume was corrected for temperature (0°C) and pressure (101.325 kPa) conditions. Gas volume (CH_4 and H_2) was obtained from the gas concentration in the gas mixture and the corrected total gas volume.

The daily amount of N in SAM (mg/d) was calculated as the product of ^{15}N enrichment in feed residues (%) and the amount of N in feed residues (mg/d) divided by the ^{15}N enrichment in N of the SAM (%) (Wang *et al*., 2000). The daily amount of N in LAM was calculated in a similar manner but using the ^{15}N enrichment and N amount in the effluent residues, and the ^{15}N enrichment in LAM. Total microbial N was quantified as the sum of N in LAM and SAM. The EMPS was defined as miligrams of microbial N per gram of OM disappeared.

The molar amount of MH was obtained from the daily production of VFA (acetate, propionate, butyrate, isovalerate and valerate), CH_4 and H_2 (Romero-Pérez *et al*., 2015b; Goel *et al*., 2009). The MH balance was obtained from the difference between MHP during fermentation and MHR_{VFA}, MHR_{CH4} and MHR_{H2} (Romero-Pérez *et al*., 2015b; Goel *et al*., 2009). The daily VFA production was estimated based on VFA molar concentration in the fermenters and effluent volume, while CH_4 and H_2 production were calculated based on the total gas production and the concentration of the corresponding gas.

Data were analysed using a mixed model procedure (SAS Inst. Inc., Cary, NC). For all variables, vessel was considered the experimental unit. For nutrient disappearance, gas production, MH balance, and fermentation variables, the model included the fixed effects of treatment, day and their interactions. For MPS and rumen microbial variables the model included the fixed effect of treatment. Apparatus was considered a random effect. Day was considered a repeated measure in the model where applicable. The variance components were estimated using the REML method and degrees of freedom were adjusted using the Kenward-Roger option. The LSD was used to determine significant differences among means. The covariance structure was selected based on the lowest Akaike and Bayesian information criteria values. Treatment significant differences and trends were declared at $P \leq 0.05$ and $P \leq 0.10$, respectively.

5.3. Results

Treatment period

The inclusion of NOP, MON or the combination of NOP and MON did not affect DM, OM, NDF or CP disappearance from the fermenters ($P \geq 0.29$; Table 5.2). Rumen liquid pH tended to increase ($P = 0.06$; Table 5.3) when NOP or NOP+MON were added. Total VFA concentration was not affected by treatments ($P = 0.54$) but molar proportion of acetate was decreased ($P < 0.01$) with addition of NOP or NOP+MON. There was no effect of treatments ($P \geq 0.31$) on molar proportion of propionate, butyrate, valerate, and isobutyrate, but molar proportion of isovalerate and caproate was increased ($P < 0.01$) with NOP and NOP+MON. The acetate to propionate ratio and the NH_3 concentration were not affected ($P \geq 0.15$) by treatments. Total gas production was decreased ($P = 0.03$; Table 5. 4) with NOP and NOP+MON. Methane production (mL/d) was decreased ($P < 0.01$) with NOP, MON and NOP+MON by 71.5, 11.8 and 69.9% respectively. When expressed as mL/g OMD, CH_4 production was also decreased by all treatments ($P < 0.01$). Methane concentration during the treatment period (Figure 5.1) was consistently decreased ($P \leq 0.01$) with NOP or NOP+MON. The addition of MON numerically decreased CH_4 concentration during d 9 to 12 of the treatment period with the greatest reduction observed on d 9 ($P \leq 0.05$) and 11 ($P \leq 0.10$). Production of H_2 was increased ($P < 0.01$) with NOP and NOP+MON by 66.4 and 82.5% respectively.

Table 5.2. Effect of 3-nitrooxypropanol (NOP), monensin (MON), and the combination of NOP with MON (NOP + MON) on *in vitro* nutrient disappearance[1].

	Treatment					
Item[2]	CON	NOP	MON	NOP + MON	SEM	*P*-value
DM	550	539	545	553	16.6	0.92
OM	545	532	543	549	17.5	0.91
NDF	219	238	219	225	18.5	0.82
CP	664	620	632	652	17.2	0.29

[1]CON, Control; NOP, 2 mg of 3-nitrooxypropanol; MON, 2 mg of monensin.

[2]g/kg of feed substrate

Table 5.3. Effect of 3-nitrooxypropanol (NOP), monensin (MON), and the combination of NOP with MON (NOP + MON) on *in vitro* rumen fermentation variables[1]

	Treatment period						Recovery period[2]					
Item	CON	NOP	MON	NOP + MON	SEM	*P*-value	CON	NOP	MON	NOP + MON	SEM	*P*-value
pH	6.94	6.98	6.95	6.97	0.01	0.06	6.94	6.95	6.93	6.95	0.01	0.70
Total VFA, mmol	36.4	34.4	38.2	35.4	1.89	0.54	36.2	35.4	35.8	38.7	2.34	0.76
VFA, mol/100 mol												
Acetate (A)	42.9^{a}	38.5^{b}	42.1^{a}	39.3^{b}	0.89	<0.01	41.9	41.7	44.6	42.0	1.32	0.25
Propionate (P)	18.1	18.4	18.0	19.7	0.73	0.37	22.2	21.9	20.2	23.1	0.75	0.10
Butyrate	19.9	20.7	19.7	19.8	0.41	0.37	18.6	17.5	18.3	16.8	0.65	0.24
Valerate	14.0	14.9	14.4	14.2	0.35	0.31	13.6	13.2	13.1	12.9	0.60	0.82
Isobutyrate	0.59	0.59	0.60	0.61	0.02	0.87	0.65^{a}	0.59^{ab}	0.57^{b}	0.61^{ab}	0.02	0.05
Isovalerate	1.23^{b}	2.24^{a}	1.46^{b}	2.05^{a}	0.13	<0.01	1.24^{b}	2.51^{a}	1.26^{b}	2.35^{a}	0.19	<0.01
Caproate	3.23^{c}	4.69^{a}	3.71^{bc}	4.38^{ab}	0.27	<0.01	1.90^{b}	2.74^{a}	1.92^{b}	2.37^{ab}	0.20	0.04
A:P ratio	2.39	2.14	2.40	2.05	0.12	0.15	1.93^{ab}	1.92^{ab}	2.23^{a}	1.83^{b}	0.09	0.04

NH_3, mmol	6.57	6.33	6.21	6.05	0.24	0.47	7.42[a]	7.75[a]	6.69[ab]	6.54[b]	0.27	0.02

[1]CON, Control; NOP, 2 mg of 3-nitrooxypropanol; MON, 2 mg of monensin.

[2]Treatments were not added during recovery period from d 16 until the end of the experiment.

[a, b, c]Within a row, means without a common superscript differ ($P < 0.05$).

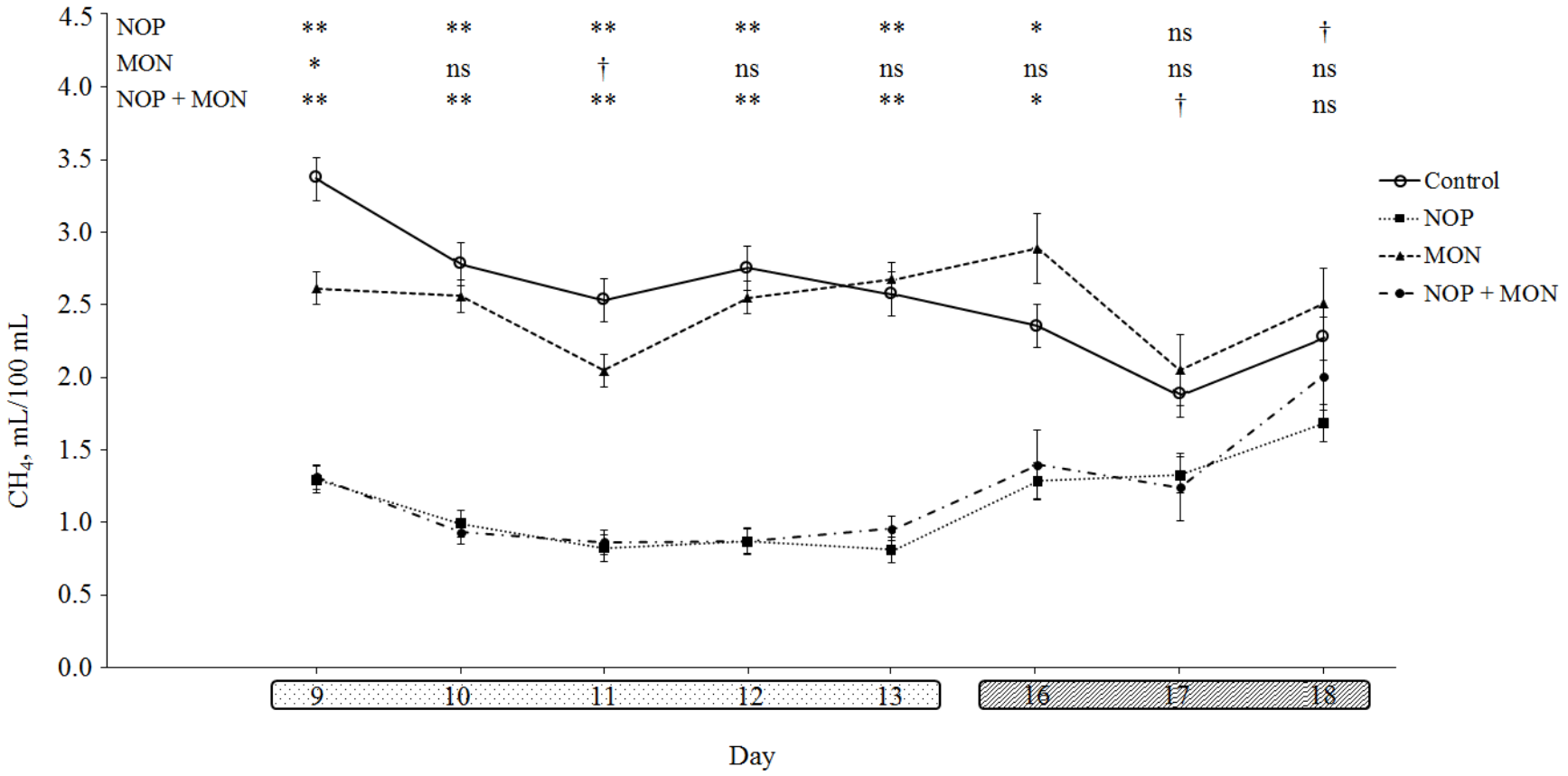

Figure 5.1. Effect of 3-nitrooxypropanol (NOP, 2 mg), monensin (MON, 2 mg), and the combination of NOP with MON (NOP + MON) compared with control on methane concentration during treatment (⬚) and recovery periods (▨). Significance of the treatment effect relative to the control is indicated for each day by ns: non-significant ($P > 0.10$); †: $P \leq 0.10$; *: $P \leq 0.05$; **: $P \leq 0.01$.

Metabolic hydrogen produced (estimated from the concentrations of acetate, propionate, butyrate, valerate and isovalerate) or recovered (estimated from the concentration of propionate, butyrate and valerate, and from CH_4 and H_2 production) was not affected by treatments ($P \geq 0.18$; Table 5.4). However, MH recovery (%), estimated from MH produced and tecovered, was decreased ($P < 0.01$) for treatments including NOP. When MHR was expressed as a molar proportion, MHR_{VFA} was increased ($P < 0.01$) by 11.2 and 10.9 units with the addition of NOP and NOP+MON respectively. The molar proportion of MHR_{CH4} was decreased ($P < 0.01$) with NOP and NOP+MON by 15.1 and 14.9, respectively, as compared to control, while the molar proportion of MHR_{H2} was increased ($P < 0.01$) by 3.95 and 4.04 with NOP and NOP+MON, respectively.

The total copy number of 16S rRNA gene for bacteria and methanogens in the liquid phase, and bacteria in the solid phase, were not affected by treatments ($P > 0.31$; Table 5.5). However, total copy number of methanogenic 16S rRNA gene in the solid phase was decreased ($P < 0.01$) with NOP and NOP+MON addition. Total microbial N, LAM, SAM, and EMPS were not affected by treatments ($P > 0.44$).

Recovery period

During the recovery period, rumen pH, total VFA, and molar proportion of acetate, butyrate and valerate were not affected by treatments ($P \geq 0.24$; Table 5.3). However, a tendency ($P = 0.10$) for decreased molar proportion of propionate was observed with NOP+MON. The molar proportion of isobutyrate was decreased ($P = 0.05$) with MON treatment. The effects observed during the treatment period for isovalerate and caproate were still present during the

recovery period ($P \leq 0.04$); additionally, the acetate to propionate ratio and the concentration of NH_3 were decreased ($P \leq 0.04$) with NOP+MON.

A tendency ($P = 0.06$) for decreased total gas production was observed with NOP and NOP+MON (Table 5.4). Methane production was still decreased by 41.6 and 37.3% with NOP and NOP+MON respectively. Methane concentration during the recovery period (Figure 5.1) was not affected by MON ($P > 0.01$). Methane concentration was still reduced by NOP and NOP+MON ($P \leq 0.05$) on d 16; however, on d 18, only a trend ($P \leq 0.10$) for decreased CH_4 production was observed for NOP treatment. Production of H_2 was no longer affected ($P = 0.53$) by treatments. Metabolic hydrogen produced or recovered and MHR_{VFA} were not affected ($P \geq 0.69$). However, the MH recovery (%) was still decreased ($P = 0.03$) with NOP and NOP+MON treatments. The effects of NOP and NOP+MON on molar proportion of MHR_{VFA} and MHR_{CH4} observed during the treatment period, persisted during the recovery period ($P \leq 0.01$). Increased ($P = 0.04$) molar proportion of MHR_{H2} was observed only for NOP treatment.

Table 5.4. Effect of 3-nitrooxypropanol (NOP), monensin (MON), and the combination of NOP with MON (NOP + MON) on *in vitro* gas production and metabolic hydrogen balance[1]

	Treatment period						Recovery period[2]					
Item	CON	NOP	MON	NOP + MON	SEM	*P*-value	CON	NOP	MON	NOP + MON	SEM	*P*-value
Gas production												
Total, L/d	1.27[a]	1.10[c]	1.24[ab]	1.14b[c]	37.2	0.03	1.14	0.98	1.22	1.03	60.9	0.06
CH_4, mL/d	36.5[a]	10.4[c]	32.2[b]	11.0[c]	1.43	<0.01	26.1[a]	15.2[b]	31.7[a]	16.3[b]	2.35	<0.01
CH_4, mL/g OMD	7.79[a]	2.32[c]	6.88[b]	2.37[c]	0.36	<0.01	---	---	---	---	---	---
H_2, mL/d	22.9[a]	38.1[b]	22.3[a]	41.8[b]	3.29	<0.01	13.1	16.2	12.7	12.5	2.16	0.53
CH_4concentration, %	2.85[a]	0.98[b]	2.65[a]	0.98[b]	0.10	<0.01	2.61[a]	1.15[b]	2.47[a]	1.22[b]	0.08	<0.01
Metabolic hydrogen												
Total produced, mmol/d	47.0	43.9	46.7	44.4	2.39	0.71	46.9	43.8	43.9	48.2	3.95	0.80
Total recovered, mmol/d	27.5	23.1	26.7	23.7	1.58	0.18	26.6	23.0	25.1	25.2	2.15	0.69
Recovery, %	58.2[a]	52.4[b]	56.6[a]	53.2[b]	0.84	<0.01	57.2[a]	51.9[b]	57.5[a]	52.0[b]	1.57	0.03
Recovered, mol/100 mol												
VFA	73.2[b]	84.4[a]	75.1[b]	84.1[a]	1.20	<0.01	79.0[b]	85.0[a]	74.7[b]	87.1[a]	1.91	<0.01
CH_4	23.4[a]	8.3[b]	21.6[a]	8.5[b]	0.89	<0.01	19.0[a]	11.5[b]	23.7[a]	10.6[b]	2.02	0.01

H_2	3.46[b]	7.41[a]	3.15[b]	7.50[a]	0.41	<0.01	2.10[ab]	2.93[a]	1.84[b]	1.99[b]	0.29	0.04

[1]CON, control; NOP, 2 mg of 3-nitrooxypropanol; MON, 2 mg of monensin.

[2]Treatments were not added during recovery period from d 16 until the end of the experiment.

[a, b, c]Within a row, means without a common superscript differ ($P < 0.05$).

Table 5.5. Effect of 3-nitrooxypropanol (NOP), monensin (MON), and the combination of NOP with MON (NOP + MON) on rumen microorganisms and microbial protein synthesis

Item	Treatment[1]				SEM	*P*-value
	CON	NOP	MON	NOP+MON		
Rumen microorganisms						
Liquid fraction, log_{10} copies/mL						
Bacteria	9.32	9.47	9.50	9.60	0.20	0.79
Methanogens	6.74	6.61	6.78	6.41	0.14	0.31
Solid fraction, log_{10} copies/g						
Bacteria	11.51	11.51	11.52	11.45	0.22	0.99
Methanogens	7.02[a]	5.83[b]	6.88[a]	5.52[b]	0.26	<0.01
Microbial N, mg/d						
Total	70.6	65.6	72.5	77.9	5.65	0.51
LAM	20.6	19.5	18.9	20.1	1.63	0.90
SAM	50.0	46.1	53.6	57.8	5.11	0.44
EMPS, mg microbial N/g OM disappeared	15.1	14.4	14.9	16.7	1.19	0.58

[1]CON, Control; NOP = 2 mg of 3-nitrooxypropanol; MON, 2 mg of monensin.

[a, b]Within a row, means without a common letter differ ($P < 0.05$).

5.4. Discussion

The forage-based diet used in this study is typical of diets fed to backgrounded beef cattle in western Canada, where barley grain and barley silage are the main feed ingredients. The rumen simulation technique was used because it simulates rumen fermentation in a controlled manner, enabling the study of compounds, such as NOP and MON, free from the influence of the host animal. The technique allows a stable fermentation to be maintained on a time-scale sufficient for microbial adaptation (Wallace *et al*., 1981).

The DMD and OMD observed in this study is in accordance with a previous *in vitro* study where different doses of NOP were evaluated using Rusitec fermenters and the same backgrounding diet (Romero-Pérez *et al*., 2015b). When dose of NOP was evaluated *in vivo* (Romero-Perez *et al*., 2014), a quadratic increase in DMD and OMD was observed. A previous meta-analysis evaluating the effects of different CH_4 antagonists *in vitro* (Ungerfeld, 2015) reported that reducing methanogenesis by more than 50% generally resulted in less fermentation and digestion in most batch cultures, but not in most continuous and semi-continuous culture sistems, such the Rusitec. The lack of reduction or the minimal reduction in feed digestibility is a remarkable characteristic observed when methanogenesis is drastically decreased through the use of inhibitors.

The use of NOP *in vivo* has typically decreased the molar proportion of acetate and increased that of propionate with concomitant reduction in the acetate to propionate ratio (Haisan *et al*., 2014; Martínez-Fernández *et al*., 2014; Romero-Pérez *et al*. 2014, 2015a). In the present experiment, the acetate proportion was decreased but the propionate proportion was not increased and the acetate to propionate ratio was not affected with NOP addition. This is in agreement with a previous *in vitro* experiment evaluating NOP dose (Romero-Pérez *et al*.,

2015b). Recently, a tendency for decreased acetate concentration with no effect on propionate concentration was observed when CH_4 was inhibited in dairy cows by NOP (de Matos *et al*., 2015). Because propionate is the most important sink of H_2 in the rumen after CH_4, an increase in propionate concentration in the rumen is usually expected when CH_4 is inhibited. However, there is a possibility for MH to be incorporated into atypical hydrogen sinks, such as H_2, formate, ethanol or microbial biomass (Ungerfeld, 2015). Indeed, accumulation of H_2 was observed in the present experiment. There is also evidence for ethanol accumulation when NOP was added to dairy cattle diets (Reynolds *et al*., 2014); however, the concentration of formate, which can become an important sink of hydrogen in the rumen when CH_4 inhibitors are utilized (Leng, 2014), has not been reported for studies evaluating NOP.

Monensin can inhibit H_2-producing bacteria, which are more apt to produce acetate and butyrate (Russell and Houlihan, 2003) and thus, a change in rumen fermentation to more propionate and less acetate was expected with MON. However, contrary to our expectation, no effect was observed for MON, and the combination of NOP plus MON did not cause a greater reduction in acetate as compared to NOP treatment alone. Additionally, no effect of MON on propionate concentrations was observed. Previous studies evaluating MON in dairy cattle consuming forage based diets (Waghorn *et al*., 2008; Grainger *et al*., 2010) did not report differences for acetate and propionate, which was in agreement with the lack of effect an effect on CH_4 in these stidies.

An increase in minor VFAs such as isovalerate and caproate with NOP has been previously observed by Romero-Perez *et al*. (2014, 2015a) and Reynolds *et al*. (2014). Caproate is an even more reduced end product of fermentation than valerate (Ungerfeld, 2015), thus it can also act as a MH sink in the rumen. Isovalerate is assumed to be formed by deamination of

leucine or isoleucine (Demeyer, 1991). However, the observed increase in isovalerate concentration with NOP is not in accordance with its lack of effect on NH_3. Additionally, the concentration of leucine and isoleucine in the rumen was not determined and consequently there is no clear explanation for increases in isovalerate with NOP.

The lack of effect of NOP on acetate during the recovery period is in agreement with a previous experiment which also evaluated NOP effects during a recovery period *in vivo* (Romero-Perez *et al*., 2014). The observation of a tendency for greater propionate proportion and decreased acetate to propionate ratio for NOP+MON during the recovery period, together with a reduction in NH_3 concentration, was unexpected. 3-Nitroxypropanol and MON are expected to increase the concentration of propionate and reduce the acetate to propionate ratio. Aditionally, MON can reduce NH_3 production, but these effects are more likely to be observed during the treatment period rather than during the recovery period as reported here. The inclusion of recovery periods, where treatment addition is discontinued, is not a common practice for experiments evaluating rumen modifiers. Our results suggest that this is a very sensitive period where carry-over effects of treatments on rumen fermentation can occur, which needs further verification.

As previously observed *in vivo* (Haisan *et al*., 2014; Martínez-Fernández *et al*., 2014; Reynolds *et al*., 2014; Romero-Perez *et al*., 2014, 2015a), and *in vitro* using the Rusitec (Romero-Pérez *et al*., 2015b), the addition of NOP decreased CH_4 production. Although the addition of MON decreased CH_4 concentration only on d 9 of the experiment, the numerical reduction observed across the treatment period for both CH_4 concentration and total gas production, resulted in a reduction in CH_4 production, confirming the reduction observed in previous Rusitec experiments (Wallace *et al*., 1981; Bogaert *et al.,* 1990; Jalč *et al*., 1992;

Wischer *et al*., 2013). However, the combination of NOP and MON did not produce an additive effect in terms of inhibition of CH_4 as hypothesized. Apparently, when rumen methanogenesis is already inhibited by NOP, there is no further inhibition from MON, even though these compounds have different modes of action. As there is no other information on the effects of NOP when combined with different antimethanogenic compounds such as MON, it may be necessary to explore possible interactions between NOP and MON using conditions where methanogenesis is not as strongly inhibited by NOP as in the present experiment.

The decrease in CH_4 caused by NOP resulted in increased H_2 production; but, this increase did not fully compensate for the reduction in CH_4. Indeed, CH_4 production was decreased from 36.5 to 10.4 mL per day (i.e., from 1.63 to 0.46 mol; 1.17 mol decrease) by NON, but H_2 production only increased from 22.9 to 38.1 mL/d (i.e., from 1.02 to 1.70 mol; 0.68 mol increase). Considering that 4 mol of H_2 are needed to synthesize 1 mol of CH_4, a 4.68 mol increase in H_2 was expected. The observed increase was only 14.5% of that expected suggesting that MH was redirected to metabolic routes not considered in the present study including formate, succinate, ethanol or microbial biomas. The elevated H_2 production observed for the control treatment was unusual but not unexpected. Although H_2 in the rumen head space rarely accumulates to concentrations greater than 1%, concentrations over this value sometimes occur (Hegarty and Gerdes, 1999). The decrease in CH_4 production was not compensated for by the increase in H_2, thus, the relative contribution of MHR_{VFA} was increased when NOP was included alone or in combination with MON, even though, the molar concentration of VFA (propionate, butyrate and valerate) in the rumen liquid was not affected. This would not have been the case if unaccounted MH sinks were considered in the balance. Therefore, the increased molar proportion of MHR_{VFA} may not imply a benefit in terms of animal production. Although most

effects observed during the treatment period were still present during the 3-d recovery period, the gradual increase in CH_4 concentration after treatment withdrawal indicates that methanogenesis inhibited in the artificial rumen is not permanently affected by NOP and it recovers relatively fast after NOP removal from the diet.

The use of ionophores *in vitro* (Ungerfeld, 2015) resulted in small numerical increases in MHR as propionate and H_2, and decreased MHR as butyrate when methanogenesis was inhibited by at least 50%. Although CH_4 production was decreased by 11.7% in the present study for MON, the MH balance for MON did not differ from control. This was probably due to the small CH_4 inhibition observed in the present study for MON as compared with results from previous meta-analysis (Ungerfeld, 2015).

The dercrease in total methanogens in the solid phase but not in the liquid phase for NOP and NOP + MON suggest that methanogen species inhabiting in the liquid phase are less susceptible to the inhibitory effect of NOP. Methanogens that cannot synthesize methyl-CoM such as *Methanobrevibacter ruminantium* have greater levels of methyl-CoM uptake compared to species that can produce it intracellularly (Balch and Wolfe, 1979; Ungerfeld., *et al*., 2004). 3-Nitrooxypropanol is a structural analog of methyl-CoM, and thus, it is possible that species able to synthesize methyl-CoM intracellularly exhibit a lower rate of transport of external CoM into the cell and therefore are more resistant to NOP. This type of resistance was previously observed for BES which is also a methyl-CoM analog (Balch and Wolfe., 1979; Ungerfeld *et al*., 2004). However, the microbial profile of rumen samples from previous beef and dairy studies (Zhou *et al*., 2015) showed a consistent increase in the proportion of *Methanobrevibacter ruminantium* when NOP was supplied, together with a decrease in the proportion of *Methanobrevibacter gottschalkii*, a methanogen which does not require external methyl-CoM to grow (Miller *et al*.,

2002). Thus, it is possible that differences in NOP susceptibility in different phases within the artificial rumen may be due to differences in the transport system. While methyl-CoM uptake by *M. ruminantium* through the cell membrane utilizes active transport (Balch and Wolfe, 1979), the system of NOP transport across the cell membrane of different rumen methanogens has not been elucidated, although it is not expected to be active (Hristov *et al*., 2015).

Based on the specific mode of action of NOP, no direct effect on rumen microbes other than methanogens was expected. However, because NOP can affect the MH balance in the rumen, as well as H_2 partial pressure, indirect effects of NOP on rumen bacteria and protozoa distribution could possibly occur, as previously observed (Zhou *et al*., 2015).

Supplementation with MON does not particularly affect methanogens; however, MON supplementation leads to the decrease in CH_4 synthesis by the inhibition of H_2 producers in the rumen, such as protozoa and Gram positive bacteria, which in turn are more apt to produce acetate than propionate (Russell and Houlihan, 2003). The reduction in CH_4 production without an effect on total methanogens or diversity in the rumen was observed when long-term supplementation with MON was assessed in dairy cattle (Hook *et al*., 2009).

The formation of CH_4 acts as a H_2 sink in the rumen increasing the ATP yield during fermentation and in turn increasing the efficiency of microbial growth, thus, inhibiting methanogenesis can result in the reduction of MPS in the rumen (Van Nevel and Demeyer, 1995). The efficiency of microbial growth is nutritionally important, because microbial protein flowing from the rumen is the most important source of protein for the ruminant (Van Nevel and Demeyer, 1995). However, as observed in the present study, NOP (Romero-Pérez *et al*., 2015b) and inhibitors such as BCM (Goel *et al*., 2009) apparently do not affect EMPS although methanogens can be drastically decreased. This is possibly because methanogens contribute only

about 1% of the total microbial matter leaving the rumen (Van Nevel and Demeyer, 1995). Lack of effect of MON addition on EMPS is in line with previous results using the Rusitec system (Li *et al* 2013, Nanon *et al*., 2014).

In conclusion, the addition of NOP and, to a smaller extent, MON, to a forage-based diet is an effective means of lowering CH_4 production *in vitro*. However, the combination of both did not produce an additive effect. When rumen methanogenesis was already inhibited by NOP, there was no further inhibition from MON. Methanogenesis in the artificial rumen was not permanently inhibited and recovered relatively fast after NOP removal from the diet. Investigation of possible interactions between NOP and MON under conditions where methanogenesis is not as strongly inhibited by NOP as in the present experiment is warranted.

5.5. Literature cited

Association of Official Analytical Chemists (AOAC) 2006. Official Methods of Analysis, 18th ed., revisions 1. AOAC International, Gaithersburg, MD, USA.

Balch, W. E., and Wolfe R. S. 1979. Transport of Coenzyme M (2-Mercaptoethanesulfonic acid) in Methanobacterium ruminantium. J. Bacteriol. 137:264–273.

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

Bogaert, C., Jouany, J. P., and Jeminet, G. 1990. Effects of the ionophore antibiotics monensin, monensin-propionate, abierixin and calcimycin on ruminal fermentations *in vitro* (Rusitec). Anim. Feed Sci. Technol. 28:183–197.

Chen, Y., Penner, G. B., Li, M., Oba, M., and Guan, L. L. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. Appl. Environment. Microbiol. 77:5770–5781.

Chen Y, Oba M, Guan L 2012. Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis Vet. Microbiol. 159:451–459.

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

de Matos, L. F., Harper, M. T., Lopes, J., Giallongo, F., Oh, J., Gruen, D., Hristov, A.N., Kindermann, M., Duval, S. 2015. Effect of 3-nitrooxypropanol on ruminal fermentation, methane and hydrogen emissions, and methane isotopic composition in dairy cows. J. Anim. Sci. 93(s3), 246 (abstract).

Demeyer, 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut. In Rumen microbial metabolism and ruminant digestion [J. P. Jouany, editor]. INRA. Paris, France.

Duffield, T. F., Rabiee, A. R., and Lean, I. J. 2008. A meta-analysis of the impact of monensin in lactating dairy cattle. Part 2. Production effects. J. Dairy Sci. 91:1347–1360.

European Medicines Agency (EMEA) 2007. Monensin (cattle, including dairy cows). EMEA/CVMP/185123/2007-Final. EMEA. London, UK.

European Medicines Agency (EMEA) 2013. European public MRL assessment report (EPMAR). Monensin (modification of MRLs). EMA/CVMP/78198/2012. London, UK.

Fricke, W. F, Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., Gottschalk, G. and Thauer, R. K. 2006.The genome sequence of *Methanosphaera stadtmanae* reveals

why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. J. Bacteriol. 188:642–658.

Goel G, Makkar, H. P., and Becker, K. 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. Br. J. Nutr. 101:1484–1492.

Grainger, C., Auldist, M. J., Clarke, T., Beauchemin, K. A., McGinn, S. M., Hannah, M. C., Eckard, R. J., and Lowe, L. B. 2008. Use of monensin controlled-release capsules to reduce methane emissions and improve milk production of dairy cows offered pasture supplemented with grain. J. Dairy Sci. 91:1159–1165.

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

Haisan, J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R., Oba, M. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

Hegarty, R. S., and Gerdes, R. 1999. Hydrogen production and transfer in the rumen. Rec. Adv. Anim. Nutr. Austr. 12:37–44.

Hook, S. E., Northwood, K. S., Wright, A. D. G., and McBride, B. W. 2009. Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. Appl. Environ. Microbiol. 75:374–380.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R. O., Kindermann, M., and Duval, S.

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 of the United Stated of America 112:10663–10668.

Immig, I., Demeyer, D., Fiedler, D., Van Nevel, C., and Mbanzamihigo, L. 1996. Attempts to induce reductive acetogenesis into a sheep rumen. Arch. Tierernaehr. 49:363–370.

Jalč, D., Baran, M., Vendrák, T. and Siroka, P. 1992. Effect of monensin on fermentation of hay and wheat bran investigated by the rumen simulation technique (RUSITEC) Arch. Tierernaehr. 42:147–152.

Kumar, S., Choudhury, P. K., Carro, M. D., Griffith, G. W., Dagar, S. S., Puniya, M., Calabro, S., Ravella, S. R., Dhewa, T., Upadhyay, R. C., Sirohi, S. K., Kundu, S. S., Wanapat, M. and Puniya, A. K. 2014. New aspects and strategies for methane mitigation from ruminants. Appl. Microbiol. Biotech. 98:31–44.

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: A paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54:519–543.

Li, Y. L., Li, C., Beauchemin, K. A. and Yang, W. Z. 2013. Effects of a commercial blend of essential oils and monensin in a high-grain diet containing wheat distillers' grains on *in vitro* fermentation. Can. J. Anim. Sci. 93:387–398.

Martínez, M. E., Ranilla, M. J., Ramos, S., Tejido, M. L., Carro, M. D. 2009. Effects of dilution rate and retention time of concentrate on efficiency of microbial growth, methane production, and ruminal fermentation in Rusitec fermenters. J. Dairy Sci. 92:3930–3938.

Martínez-Fernández, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martín-García, A. I., Molina-Alcaide, E., Kindermann, M., Duval, S., and Yáñez-Ruiz, D. R. 2014. Effects of

ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. J. Dairy Sci. 97:3790–3799.

Miller, T. L., and Lin, C. 2002. Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. Int. J. Syst. Evol. Micr. 52:819–822.

Nanon, A., Suksombat, W., Beauchemin, K. A., and Yang, W. Z. 2014. Assessment of lemongrass oil supplementation in a dairy diet on *in vitro* ruminal fermentation characteristics using the rumen simulation technique. Can. J. Anim. Sci. 94:731–736.

Prakash, D. 2014. Methyl-coenzyme M reductase: Elucidating the process of activation and study of the effect of the methanogenesis inhibitor 3-nitrooxypropanol. Doctoral dissertation. Auburn University. Auburn, Alabama, USA. https://etd.auburn.edu/handle/10415/4463.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J. Dairy Sci. 97:3777–3789.

Rhine, E. D., Sims, G. K., Mulvaney, R. L., and Pratt, E. J. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Sci. Soc. Amer. J. 62:473–480.

Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682–4693.

Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2015a. Sustained reduction in methane

production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. J. Anim. Sci. 93:1780–1791.

Romero-Pérez, A., Okine, E. K., Guan, L. L., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2015b. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). Anim. Feed Sci. Technol. 209:98–109.

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

Tomkins, N. W., Colegate, S. M., and Hunter, R. A. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49:1053–1058.

Ungerfeld, E. M., Rust, S. R., Boone, D. R., and Liu, Y. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. J. Appl. Microbiol. 97:520–526.

Ungerfeld, E. M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6(37):1–17.

Van Nevel, C., and Demeyer, D. 1995. Feed additives and other interventions for decreasing methane emissions. In Biotechnology in animal feeds and animal feeding pp. 329–349. [R. J., Wallace, A. Chesson, editors] VCH Verlagsgesellschaft mbH., Weinheim, Germany.

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

Waghorn, G. C., Clark, H., Taufa, V., and Cavanagh, A. 2008. Monensin controlled-release capsules for methane mitigation in pasture-fed dairy cows. Austr. J. Exp. Agric.48:65–68.

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

Wischer, G., Boguhn, J., Steingaß, H., Schollenberger, M., Hartung, K., and Rodehutscord, M. 2013. Effect of monensin on *in vitro* fermentation of silages and microbial protein synthesis. Arch. Anim. Nutr. 67:219–234.

Zhou, M., Hernández-Sanabria, E., and Guan, L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Appl. Environm. Microbiol. 75:6524–6533.

Zhou, M., Chen, Y., Haisan, J., Romero-Perez, A., Beauchemin, K. A., Oba. M., Kindermann, M., Duval, S., and Guan, L. L. 2015. Effects of feeding 3-nitrooxypropanol (NOP) on rumen microbial profiles in lactating dairy cows and beef cattle. Proceedings of the ADSA-ASAS Joint Annual Meeting, July 12-16, 2015, Orlando, Florida, USA.

6. General discussion and final conclusion

6.1. General discussion

The novel compound NOP was designed as a structural analog of methyl-CoM, and proposed to reduce CH_4 production by inhibiting MCR. Therefore, it was hypothesized that NOP would reduce CH_4 production from beef cattle without detrimental effects on ruminal fermentation. Additionally, it was hypothesized that use of NOP together with MON would further reduce CH_4 production because of the different modes of action. To test these hypotheses, four experiments were conducted. Two animal experiments were designed to evaluate the potential of NOP to reduce CH_4 emissions using different doses (Chapter 2) and establish if reduction in CH_4 production with NOP is sustained over time (Chapter 3). Animal experiments were conducted using metabolic chambers, a technique with the ability to make accurate and precise measurements of emissions from cattle (Johnson and Johnson, 1995). Additionally, two *in vitro* experiments using Rusitec fermenters were conducted to determine the optimum NOP dose for this system, to look further into the MH balance (Chapter 4) and to evaluate possible interactions between NOP and the well-known feed additive MON (Chapter 5). Methane production, the main variable of interest, was consistently decreased in beef cattle and in Rusitec fermenters.

By conducting a series of *in vivo* and *in vitro* studies, it was possible to examine the effects of greater doses of NOP on CH_4 and digestibility, without risk of toxicity to animals. Furthermore the in vitro study was conducted to explore the effects of NOP on MH dynamics and MPS. To compare the results from the *in vitro* and *in vivo* studies, it is important to consider the relative doses of NOP used. In the first *in vivo* study (Chapter 2), the dose ranged from 0 to 4.5 mg NOP/kg BW, with the highest dose equivalent to 2.8 g/d for animals consuming an

average of 11.6 kg/d DMI. Thus, the dietary concentration of NOP ranged from 0 to 241 mg NOP/kg DM. The second *in vivo* study (Chapter 3) used a concentration of 280 mg NOP/kg DM. In the first *in vitro* study (Chapter 4), the dose ranged from 0 to 20 mg NOP/10 g DM equivalent to 0 to 2,000 mg NOP/kg DM. Thus, the higest dose used was approximately 7 to 8-times greater than the highest dose used in both *in vivo* studies. In the second *in vitro* study (Chapter 5), the concentration of NOP was 200 mg NOP/kg DMI, which was similar to the highest dose used in both *in vivo* studies.

In the first animal experiment (Chapter 2), a linear dose response to NOP was established. Increasing NOP dose (0, 0.75, 2.25, and 4.5 g NOP/kg BW, equivalent to 0, 0.5, 1.4, and 2.8 g/d) decreased CH_4 production linearly, with the highest dose significantly different from the control (33% reduction). Thus it was established that the effect of NOP is dose dependent. Similarly, a linear reduction in CH_4 production was evident when increasing doses of BCM were added to the diet of sheep (Sawyer *et al*., 1974) and beef (Tomkins *et al*., 2009). Recently, the dose response effect of NOP observed in the present research was confirmed in dairy cattle (Hristov *et al*., 2015).

The 33% reduction obtained in beef at the the highest dose observed in Chapter 2 was comparable with the average 22% reduction observed in sheep (Martínez-Fernández *et al*., 2014). Although NOP dose (calculated in the same units) used in beef (217 mg NOP/kg DMI) was twice the dose used in sheep (111 mg NOP/kg DMI), CH_4 reduction in beef was not twice that observed in sheep. In contrast, the 33% reduction observed for the highest dose in beef (Chapter 2) was half the 60% reduction observed in dairy cows (Haisan *et al*., 2014) even though the NOP dose used in beef (217 mg NOP/kg DMI) was 67% greater than dose used in dairy (130 mg NOP/kg DMI). The difference in the magnitude of CH_4 reduction among studies using NOP

suggests that, in addition to dose, the mode of providing NOP (mixed with the diet, added through the rumen cannula or top dressed on the feed) affects CH_4 reduction potential. Mixing NOP with the diet as was performed in Chapter 3 may have improved its effectiveness by synchronizing the availability of the compound in the rumen with ruminal fermentation. This speculation is supported by the drastic reduction in CH_4 production observed during the first 2 h after feeding with NOP addition and the rapid increase in CH_4 after this time, suggesting that NOP is rapidly absorbed, metabolized, and/or washed out from the rumen with a pulse dose (Reynolds *et al*., 2014).

The observed reduction in acetate concentration and increase in propionate, butyrate and valerate concentrations with NOP use in Chapter 2 indicates a redirection of MH not used for CH_4 synthesis to other MH sinks. A similar shift in rumen fermentation is commonly observed with CH_4 inhibitors such as BES (Immig *et al*., 1996) and BCM (Knight *et al*., 2011; Abecia *et al*., 2012; Mitsumori *et al*., 2012) and was also observed for NOP in other studies (Haisan *et al*., 2014; Martínez-Fernández *et al*., 2014).

Inhibitors have drastically decreased CH_4 production (i. e., > 90%) with no effect on digestibility (Johnson *et al*., 1972; Knight *et al*., 2011; Mitsumori *et al*., 2012) and thus, the lack of negative effect on digestibility observed with NOP in Chapter 2 is in accordance with previous studies. The reduction of CH_4 production by means of inhibitors can result in increased H_2 production (Table 1.2) which in turn may reduce feed digestibility due to the negative feedback that H_2 exerts on the regeneration of cofactors needed for feed fermentation by rumen microbes (Leng, 2014). Instead of H_2, formate can be produced within biofilms, which would have no effect on the partial pressure of H_2 and consequently on digestibility. Formate may diffuse towards the rumen fluid and be converted to H_2 and CO_2 (Leng, 2014). Although NOP

addition resulted in increased H_2 production (1.27 g/d), this represented just a small proportion (3%) of that expected due to the decrease in CH_4 (Hristov *et al*., 2015). The possible adaptation of rumen microbes to increased H_2 in the rumen (Hristov *et al*., 2015) may also contribute to the lack of NOP effect on feed digestion.

3-Nitrooxypropanol was designed to directly affect the last step of methanogenesis (Prakash *et al*., 2015), thus a reduction in total methanogens was expected. However, the lack of effect on methanogens observed in Chapter 2 indicates that metabolic activity rather than the total methanogenic population itself were responsible for CH_4 reduction.

Long-term (112 d) addition of NOP to beef cattle consuming a backgrounding diet (Chapter 3) resulted in sustained reduction of CH_4 production by 59% with no signs of adaptation. This was the first study to examine whether animals fed NOP adapt over time. Sustained reduction observed in the long-term study was recently confirmed when NOP was added to the diet of dairy cattle during 12 consecutive weeks (Hristov *et al*., 2015).

The magnitude of CH_4 reduction in Chapter 3 was comparable to the 60% reduction observed in dairy cattle by Haisan *et al*. (2014), confirming that mixing NOP with feed (as was done in Chapter 3) may improve the CH_4 inhibitory potential of NOP as compared to top dressing NOP on feed (Chapter 2) or dosing NOP through a rumen cannula (Martínez-Fernández *et al*., 2014).

In the long-term study, CH_4 reduction was accompanied by reduction in acetate proportion and increases in propionate, butyrate and valerate proportions, confirming the results from the first in vivo study (Chapter 2). Stoichiometric calculation showed that NOP addition decreased the amount of MH used for CH_4 synthesis, and partially redirected MH to VFA synthesis (propionate, butyrate, valerate). However, the increase in MH used for VFA did not

fully account for the reduction in MH used for CH_4 synthesis indicating a redirection of MH to undetermined sinks. The reduction in methanogens with NOP addition was in agreement with the reduction in CH_4 production and suggested that a substantial reduction in CH_4 emissions may partially occur as a result of a decreased methanogen population. Methane production and most variables affected by NOP were restored to control levels during the recovery period.

As discussed above, decreased CH_4 production observed in Chapter 2 was not fully compensated by increased VFA (propionate, butyrate and valerate) and suggested accumulation of H_2 in the rumen. However this hypothesis was not possible to prove *in vivo* (Chapter 2 and 3) because the H_2 measurement technique was not established in our metabolic chambers. Therefore, *in vitro* experiments (Chapters 4 and 5) were designed to look further into the fate of MH in the methanogenesis-inhibited rumen. The rumen simulation technique was selected because it mimics rumen fermentation in a controlled manner, enabling the study of compounds such as NOP, free from the influence of the host animal. Using Rusitec fermenters allowed us to examine aspects, such as MH balance, and microbial protein synthesis that were not assessed *in vivo*.

Feeding NOP in Rusitec fermenters decreased CH_4 production by up to 86% with the highest dose (20 mg/d) (Chapter 4). This is a larger reduction compared to the maximum 59 and 60% reduction observed in beef (Chapter 3) and dairy (Haisan *et al*., 2014) cattle and was mainly due to the larger doses used *in vitro*. Larger doses used *in vitro* allowed us to evaluate possible negative effects of NOP on feed digestibility. Doubling NOP dose from 5 to 10 mg/d and from 10 to 20 mg/d did not decrease CH_4 production to the same magnitude. This indicates a point along a curve of increasing NOP dose where there may not be further significant CH_4 reduction with an additional increment. Although CH_4 production did not differ among levels of NOP (5,

10 or 20 mg/d), a linear response was observed. This is in agreement with the linear dose response for NOP observed *in vivo* (Chapter 2). In the second *in vitro* study, with a dose similar to that used in vivo, the 72% decrease in CH_4 was more than double that observed in the first *in vivo* study (32% decrease), where animals were fed *ad libitum*. The diference in the relative decrease in CH_4 prodiction *in vivo* and *in vitro* was likely due to differences in passage rate. The passage rate of fluid within animals fed *ad libitum* would likely have been greater than the 2.9%/h passage rate used *in vitro*. Addtionally, there may be absorption of the compound *in vivo*, which would not be the case *in vivo*. The pH in vivo was lower than the pH *in vitro*.

Typically, the use of CH_4 inhibitors does not negatively affect feed digestibility. In fact, digestibility increased with use of inhibitors in beef (Chapter 2), dairy (Hristov *et al*., 2015) and sheep (Sawyer *et al*., 1974) studies. Thus the lack of negative effecs of NOP on digestibility when evaluated using Rusitec fermenters was in agreement with *in vivo* experiments.

The reduction in acetate proportion without increase in propionate proportion was not expected. Methane inhibitors are well known to increase propionate proportion; however, this may not happen in continuous cultures due to lack of adaptation of some propionate producers (Ungerfeld, 2015). It was demonstrated that decreased CH_4 production was partially accounted for by increased H_2 production; however, the reduction in MH recovery percent indicated that MH was also redirected to unaccounted sinks.

Methanogens inhabiting the liquid phase of the Rusitec fermenters were not affected by NOP, in contrast to those associated with the solid phase. This indicates that methanogens present in the liquid phase may be less sensitive to NOP. Sensitivity to NOP could be related to differences in NOP intake mechanism among methanogen species. It was demonstrated that methanogens that are able to produce methyl-CoM intracellularly have lower BES intake (Balch

and Wolfe, 1979; Ungerfeld *et al*., 2004). 3-Nitroxypropanol and BES have similar chemical structure and thus the level of NOP intake is expected to be different between methanogens that can or cannot produce methyl-CoM.

Eventual NOP approval for animal use may make it essential to evaluate possible interactions between NOP and feed additives that are commonly used in animal production such as MON. Rusitec fermenters were used to examine NOP (2 mg/d), MON (2 mg/d) and their combination. The antimethanogenic effects of NOP and MON were confirmed in the artificial rumen. Methane production was decreased by 72 and 12% with NOP and MON respectively; however, the combination of NOP and MON did not have additive effects. When rumen methanogenesis is already inhibited by NOP, there is no further inhibition from MON. Thus, investigation of possible interactions between NOP and MON under conditions where methanogenesis is not as strongly inhibited by NOP as in the present experiment is warranted. Monensin increases feed efficiency (Russell and Houlihan, 2003) and thus, possible interaction of NOP and MON would be more evident *in vivo* given that increased feed efficiency would also contribute to reductions in CH_4 production per unit of feed. The lack of effect of NOP on nutrient digestibility and propionate proportion previously observed (Chapter 1) was confirmed; however, the lack of effect of MON on most evaluated variables except for CH_4 production was not expected. Typically, supplementation with MON reduces CH_4 synthesis by the inhibition of H_2 producers in the rumen, with consequent reduction in acetate and increment in propionate proportions (Russell and Houlihan, 2003), and thus the observations for MON needs further verification.

Methane inhibition by NOP resulted in increased H_2 gas production; however, this increase was only 14.5% of that expected suggesting that MH was redirected to other sinks. As

observed in the first Rusitec experiment (Chapter 4), methanogens in the solid phase were decreased while methanogens in the liquid phase were not affected, indicating that methanogens in the liquid phase are less sensitive to NOP probably due to differences in NOP intake and type of NOP transport through the cell membranes. Although CH_4 production was considerably decreased with NOP alone or in combination with MON, CH_4 concentration recovered relatively quickly (3 days) after treatment removal from the diet. This was in agreement with the long-term beef study (Chapter 3) and confirms that NOP addition does not permanently affect CH_4 production.

6.2. Future research

The present thesis demonstrated the effectiveness of NOP to reduce CH_4 production *in vitro*, using Rusitec fermenters, and *in vivo* when feed to beef cattle consuming backgrounding diets. The persistency of the inhibitory effect of NOP on CH_4 production was also demonstrated over a long-term feeding period without signs of microbial adaptation. The positive results obtained from our research are encouraging and open the possibility of continued investigation of this compound. The decrease in CH_4 production observed in the *in vivo* experiments (Chapters 2 and 3) together with increased propionate proportion, a small reduction in DMI and no effect on BW suggest that NOP may positively affect animal productivity; however, this needs to be confirmed in long terms studies. Dairy cattle fed NOP increased BW gain indicating that increased metabolizable energy due to CH_4 reduction was used for BW gain (Haisan *et al*., 2014; Hristov *et al*., 2015). The adoption of specific CH_4 mitigation strategies will depend to a great extent on the ability of such strategies to provide benefits in addition to CH_4 mitigation. Most

importantly, studies need to confirm effects on animal production, and thus the effects of NOP on animal performance should be evaluated under farm conditions.

Experiments evaluating NOP have used diets within a very narrow range of forage concentration in the diet (i.e., from 38 to 60% forage on a DM basis; Haisan *et al*., 2014; Romero-Perez *et al*., 2014). The effectiveness of NOP needs to be confirmed across diets with different forage to concentrate proportions. Previous studies suggest that dietary forage content can influence the effects of rumen fermentation modifiers such as MON on CH_4 production in cattle (Guan *et al*., 2006). Determining methanogen species sensitive to NOP, but also possible effects on bacteria or protozoa species is necessary to better understand the effects of NOP on rumen fermentation. The effect of NOP on total methanogens, bacteria and protozoa in the rumen has not been consistent. Total methanogens were decreased in studies by Haisan *et al*. (2014) and in the beef study reported in Chapter 3; however, no effect on methanogens was reported in Chapter 2. It is possible that particular species and metabolic activity rather than total methanogens may be responsible for CH_4 production in some particular conditions. Future studies must be specifically designed to provide insight into this area to determine the microbial species affected by NOP. Additionally, the effectiveness of NOP should also be evaluated in grazing cattle.

Methane emited from manure produced by animals consuming NOP needs to be evaluated. A reduction in CH_4 production from manure of NOP treated animals should be expected if part of the consumed NOP is excreted in feces or if methanogens in feces are still inhibited. Although a reduction in CH_4 emissions from feces is desirable in most scenarios, this would represent a problem if feces from NOP treated animals are employed as substrate for CH_4 production using biodigestors due to the need for maximizing CH_4 synthesis in such systems.

The evaluation of combined effects of NOP with other CH_4 mitigation strategies to determine possible additive or antagonistic effects is warranted. *In vitro* experiments evaluating all possible MH sinks are necessary to better assess the MH balance in the NOP inhibited rumen. Additionally, *in vivo* experiments evaluating NOP should measure H_2 gas production, dissolved H_2 in the rumen liquid and other MH sinks (such as formate, succinate, ethanol, aldehydes, microbial biomass, and others) to track the fate of MH not used for CH_4 synthesis. 3-Nitrooxypropanol is a novel compound and the experimental conditions under which NOP can be evaluated are numerous; thus, further studies are needed to confirm the effects reported in this thesis for beef cattle. For example, the studies conducted in this research focused on cattle fed growing (backgrounding) diets, but the effects of NOP may be dependant on the forage to concentrate ratio, the ingredient compostion of the diet, and so forth.

6.3. Industry perspective

3-Nitrooxypropanol represents one of the most promising CH_4 mitigation strategies, given its elevated antimethanogenic potential and long-term effectiveness. However, factors other than inhibitory potential and persistency must be addressed before eventual NOP approval for animal use and on-farm adoption. For registration purposes, NOP needs to be proven to be safe for humans, animals and environment. It is essential that NOP does not accumulate (as NOP or as a metabolite) in the tissues (e.g., meat) or secretions (e.g., milk) of supplemented animals. For on-farm adoption, a number of important conditions are needed including: NOP needs to be easy to implement; it should improve animal performance; and it needs to be produced and commercialized at a cost compatible with the animal nutrition industry (Duval and Kindermann, 2012). It is unlikely that farmers will adopt mitigation strategies that require additional investment, or which have no production or economic benefits or that are not mandatory or supported by government (Hristov *et al*., 2014). The synthetic nature of NOP could be a barrier for adoption due to public perception and existent or current regulations (Hristov *et al*., 2014). However, a compound such as NOP that reduces the carbon footprint from animal agriculture may also generate a positive public perception. The use of NOP is more likely to be adopted in intensive production systems in which animals consume feed directly in a feeder and preferably when animals consume total mixed rations. It is likely that the use of NOP in extensive production systems, such as grazing animals, would be limited unless technologies can be developed to dose NOP to pastured cattle.

6.4. Final conclusions

The research demonstrated that NOP addition is an effective means of lowering CH_4 production from beef cattle consuming a high forage (i.e., backgrounding) diet. Methane reduction by means of NOP addition was dose dependent and was maximized when NOP was mixed with the feed, probably due to a better synchronization between NOP concentration in the rumen and rumen fermentation. Digestibility was not affected by NOP addition; however, DMI was decreased slightly. Overall, addition of NOP affected rumen fermentation by decreasing acetate and increasing propionate proportions, which could be beneficial for animal production. Hydrogen not used for CH_4 synthesis when NOP was included was partially redirected to H_2 gas production, which also represents a loss of feed energy. The combination of NOP and MON was not additive; but nor were they antagonistic. This finding is important because MON is currently used extensively in feedlot cattle diets in North America. Methanogenesis was not permanently inhibited by NOP; rather it recovered relatively fast after NOP removal from the diet. These findings provide substantial knowledge on the use of the novel compound NOP as an inhibitor of rumen methanogenesis in beef cattle and contribute to the better understanding of the feed fermentation process in the inhibited rumen.

6.5. Literature cited

Abecia, L., Toral, P.G., Martín-García, A. I., Martínez, G., Tomkins, N. W. Molina-Alcaide, E., Newbold, C. J., and Yañez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95:2027–2036.

Balch, W. E., and Wolfe, R. S. 1979. Transport of coenzyme m (2-mercaptoethanesulfomc acid) in Methanobacterium ruminantium. J. Bacteriol. 137:264–237.

Duval S, and Kindermann, M. 2012. Use of nitrooxy organic molecules in feed for reducing enteric methane emissions in ruminants, and/or to improve ruminant performance. World Intellectual Property Organization. International Patent Application WO 2012/084629 A1.

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

Haisan, J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R., Oba, M. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

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

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R. O., Kindermann, M., and Duval, S. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. U. S. A. 112:10663–10668.

Immig, I., Demeyer, D., Fiedler, D., Van Nevel, C., and Mbanzamihigo, L. 1996. Attempts to induce reductive acetogenesis into a sheep rumen. Arch. Tierernaehr. 49:363–370.

Johnson, E. D., Wood, A. S., Stone, J. B., and Moran Jr., E. T. 1972. Some effects of methane inhibition in ruminants (steers). Can. J. Anim. Sci. 52:703–712.

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

Knight, T., Ronimus, R. S., Dey, D., Tootill, C., Naylor, G., Evans, P., Molano, G., Smith, A., Tavendale, M., Pinares-Patiño, C. S., and Clark, H. 2011. Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. Anim. Feed Sci. Technol. 166–167:101–112.

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

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., and McSweeney, C. S. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108:482–491.

Reynolds, C.K., Humphries, D.J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of incremental rumen doses of 3-nitrooxypropanol on methane production, digestion and rumen parameters in lactating dairy cows. J. Dairy Sci. 97:3777–3789.

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

Sawyer, M. S., Hoover, W. H., and Sniffen, C. J. 1974. Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. J Anim. Sci. 38:908–914.

Tomkins, N. W., Colegate, S. M., and Hunter, R. A. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49:1053–1058.

Ungerfeld, E. M. Rust, S. R., Boone, D. R., and Liu, Y. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. J. Appl. Microbiol. 97:520–526.

Ungerfeld, E.M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6(37):1–17.

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

LITERATURE CITED

Abecia, L., Toral, P. G., Martín-García, A. I., Martínez, G., Tomkins, N. W. Molina-Alcaide, E., Newbold, C. J., and Yañez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95:2027–2036.

Andries, J. I., Buysse, F. X., De Brabander, D. L., and Cottyn, B.G. 1987. Isoacids in ruminant nutrition: their role in ruminal and intermediary metabolism and possible influences on performances - A review. Anim. Feed Sci. Technol. 18:169–180.

Alexandratos, N., and J. Bruinsma. 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working paper No. 12-03. Rome, FAO.

Animut, G., Puchala, R., Goetsch, A. L., Patra, A. K., Sahlu, T., Varel, V. H. and Wells, J., 2008. Methane emission by goats consuming diets with different levels of condensed tannins from Lespedeza. Anim. Feed Sci. Technol. 144:212–227.

AOAC. 2005. Official methods of analysis. 18th ed. AOAC Int., Gaithersburg MD.

AOAC. 2006. Official methods of analysis, 18th ed., revisions 1. AOAC Int., Gaithersburg, MD.

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

Bannink, A., and Tamminga S. 2005. Rumen Function. In Quantitative aspects of ruminant digestion and metabolism, 2nd ed., pp. 263-268 [J. Dijkstra, J.M. Forbes, J. France, editors]. CABI Publishing. Cambridge, MA. USA.

Bauchop, T. 1967. Inhibition of rumen methanogenesis by methane analogues. J. Bacteriol. 94:171–175.

Balch, W. E., and Wolfe, R. S. 1979. Transport of coenzyme M (2-mercaptoethanesulfomc acid) in *Methanobacterium ruminantium*. J. Bacteriol. 137: 264–237.

Bayaru, E., Kanda, S., Kamada, T., Itabashi, H., Andoh, S., Nishida, T., Ishida, M., Itoh, T., Nagara, K., and Isobe, Y. 2000. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. Anim. Sci. J. 72:139-146.

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

Beauchemin, K. A., McGinn, S. M. Martinez, T. F., and T. A. McAllister. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85:1990–1996.

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

Belanche, A., de la Fuente G., and Newbold C. J. 2014. Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiol. Ecol. 90:663–677.

Bogaert, C., Jouany, J. P., and Jeminet, G. 1990. Effects of the ionophore antibiotics monensin, monensin-propionate, abierixin and calcimycin on ruminal fermentations *in vitro* (Rusitec). Anim. Feed Sci. Technol. 28:183–197.

Burrin, D. G., and Britton, R. A. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63:888–893.

Canadian Council on Animal Care. 1993. Guide to the care and use of experimental animals. Vol. 1. 2nd ed. [E.D. Olfert, B. M. Cross, and A. A. McWilliam, editors]. CCAC, Ottawa, ON, Canada.

Canadian Council on Animal Care. 2009. CCAC guidelines on: The care and use of farm animals in research, teaching and testing. Ottawa, ON, Canada. http://www.ccac.ca/Documents/Standards/Guidelines/Farm_Animals.pdf. (Accessed February 10, 2015.)

Cersosimo, L. M., and Wright A. D. G. 2015. Rumen methanogens. In Rumen microbiology: From evolution to revolution [A. K. Puniya, R. Singh, D. N. Kamra, editors]. Springer. New Delhi, India.

Chalupa, W. 1997. Manipulating rumen fermentation. J. Anim. Sci. 46:585–599.

Chen, Y., Penner, G. B., Li, M., Oba, M., and Guan, L. L. 2011.Changes in Bacterial Diversity Associated with Epithelial Tissue in the Beef Cow Rumen during the Transition to a High-Grain Diet.

Chen, Y., Oba, M., and Guan, L. 2012. Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. Vet. Microbiol. 159:451–459.

Chung, Y.-H., Walker, N. D., McGinn, S. M., and Beauchemin, K. A. 2011. Differing effects of 2 active dried yeast (*Saccharomyces cerevisiae*) strains on ruminal acidosis and methane production in nonlactating dairy cows. J. Dairy Sci. 94:2431–2439.

Chung, Y.-H., Zhou, M., Holtshausen, L., Alexander, T. W., McAllister, T. A., Guan, L. L., Oba, M., and Beauchemin, K. A. 2012. A fibrolytic enzyme additive for lactating Holstein cow

diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. J. Dairy Sci. 95:1419–1427.

Cieslak, A., Szumacher-Strabel, M., Stochmal, A., and Oleszek, W. 2013. Plant components with specific activities against rumen methanogens. Animal 7:253–265.

Czerkawski, J. W., and Breckenridge, G. 1975a. New inhibitors of methane production by rumen micro-organisms. Development and testing of inhibitors *in vitro*. Br. J. Nutr. 34:429–446.

Czerkawski, J. W., and Breckenridge, G. 1975b. New inhibitors of methane production by rumen micro-organisms. Experiments with animals and other practical possibilities. Br. J. Nutr. 34:447–57.

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

DasSarma, S., J.A. Coker, and P. DasSarma. 2009. Archaea - Overview. In Desk Encyclopedia of Microbiology, 2nd edition, [M. Schaechter, editor] Academic Press p. 118-139.

Demeyer, 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut. In Rumen microbial metabolism and ruminant digestion [J. P. Jouany, editor]. INRA. Paris, France.

Dehority, B. A.. 1993. Laboratory manual for classification and morphology of ruminal ciliate protozoa. CRC Press, Boca Raton, FL.

de Matos, L. F., Harper, M. T., Lopes, J., Giallongo, F., Oh, J., Gruen, D., Hristov, A. N., Kindermann, M., and Duval, S. 2015. Effect of 3-nitrooxypropanol on ruminal fermentation, methane and hydrogen emissions, and methane isotopic composition in dairy cows. J. Anim. Sci. 93(s3):246.

Dong, Y., Bae, H. D., McAllister, T. A., Mathison, G. W., and Cheng, K.-J. 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.

Duffield, T. F., Rabiee, A. R., and Lean, I. J. 2008. A Meta-Analysis of the Impact of Monensin in Lactating Dairy Cattle. Part 2. Production Effects. J. Dairy Sci. 91:1347–1360.

Duval, S., and Kindermann, M. 2012. Use of nitrooxy organic molecules in feed for reducing enteric methane emissions in ruminants, and/or to improve ruminant performance. World Intellectual Property Organization. International Patent Application WO 2012/084629 A1.

de Bok, F. A. M., Plugge, C. M., and Stams, A. J. M. 2004. Interspecies electron transfer in methanogenic propionate degrading consortia. Water Res. 38:1368–1375.

Dohme, F., Machmüller, A., Wasserfallen, A., and Kreuzer, M. 2001. Ruminal methanogenesis as infuenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32:47–51.

Environment Canada, 2015. National Inventory Report 1990–2013. Greenhouse gas sources and sinks in Canada, The Canadian Government's Submission to the UN Framework Convention on Climate Change, Part 1.

EPA. 2010. Methane and Nitrous Oxide Emissions from Natural Sources . U.S. Environmental Protection Agency, Washington, DC, USA.

EPA. 2012. Global anthropogenic non-CO_2 greenhouse gas emissions: 1990 – 2030. EPA 430-R-12-006. U.S. Environmental Protection Agency. Office of Atmospheric Programs, Climate Change Division. Washington, DC.

Ermler, U., Grabarse, W., Shima, S., Goubeaud, M., and Thauer, R. K. 1997. Crystal structure of methyl-coenzyme M reductase: the key enzyme of biological methane formation. Science. 278:1457–62.

European Medicines Agency (EMEA). 2007. Monensin (Cattle, including dairy cows). EMEA/CVMP/185123/2007-Final. EMEA, London UK.

European Medicines Agency (EMEA) 2013. European public MRL assessment report (EPMAR). Monensin (modification of MRLs). EMA/CVMP/78198/2012. London, UK.

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

France, J. and Dijkstra, J. 2005. Volatile fatty acid production. In Quantitative aspects of ruminant digestion and metabolism, 2nd ed., pp. 156-175 [J. Dijkstra, J.M. Forbes, J. France, editors]. CABI Publishing. Cambridge, MA. USA.

Fraser, G. R., Chaves, A. V., Wang, Y., McAllister, T. A., Beauchemin, K. A., and Benchaar, C. 2007. Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems. J. Dairy Sci. 90:2315–2328.

Fricke, W. F, Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., Gottschalk, G. and Thauer, R. K. 2006.The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. J. Bacteriol. 188:642–658.

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

Goel, G., Makkar, H. P. S., and Becker, K. 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. Br. J. Nutr. 101:1484–1492.

Goel, G., and Makkar, H. P. S. 2012. Methane mitigation from ruminants using tannins and saponins. Trop. Anim. Health. Prod. 44:729–739.

Golden, R. J., Holm, S. E., Robinson, D. E., Julkunen, P. H., and Reese, E. A., 1997. Chloroform mode of action: implications for cancer risk assessment. Reg. Toxicol. Pharm. 26:142–155.

Grainger, C., Auldist, M. J., Clarke, T., Beauchemin, K. A., McGinn, S. M., Hannah, M. C., Eckard, R. J., and Lowe, L. B. 2008. Use of monensin controlled-release capsules to reduce methane emissions and improve milk production of dairy cows offered pasture supplemented with grain. J. Dairy Sci. 91:1159–1165.

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

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

Gunsalus, R. P., Romesser, J. A., and Wolfe, R. S. 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum.* 17:2374–2377.

Grainger, C., Auldist, M. J., Clarke, T., Beauchemin, K. A., McGinn, S. M., Hannah, M. C. Eckard, R. J., and Lowe, L. B. 2008. Use of monensin controlled-release capsules to

reduce methane emissions and improve milk production of dairy cows offered pasture supplemented with grain. J. Dairy Sci. 91:1159–1165.

Grainger, C., Clarke, T., Auldist, M. J., Beauchemin, K. A., McGinn, S. M., Waghorn, G. C., and Eckard, R. J. 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 89:241–251.

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

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

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

Haisan, J., Sun, Y., Beauchemin, K., Guan, L., Duval, S., Barreda, D. R., and Oba, M. 2013. Effects of feeding 3-nitrooxypropanol, at varying levels, on methane emissions and rumen fermentation in lactating dairy cows. Adv. Anim. Biosci. 4:326 (Abstr.).

Haisan, J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R., and Oba, M., 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

Hegarty, R. S., Gerdes, R., 1999. Hydrogen production and transfer in the rumen. Rec. Adv. Anim. Nutr. Austr. 12:37–44.

Hristov, A. N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J., and

Oosting, S. 2013a. In Mitigation of greenhouse gas emissions in livestock production – A review of technical options for non-CO_2 emissions. [P. J. Gerber, B. Henderson, H.P.S. Makkar, editors]. FAO Animal Production and Health Paper No. 177. FAO, Rome, Italy.

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

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R. O., Kindermann, M., and Duval, S. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. U. S. A. 112:10663–10668.

Holtshausen, L., Chaves, A. V., Beauchemin, K. A., McGinn, S. M., McAllister, T. A., Odongo, N. E., Cheeke, P. R., and Benchaar, C. 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows, J. Dairy Sci. 92:2809–2821.

Hook, S. E., Wright, A. G., and McBride, B. W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea. 2010:1–11.

Hulshof, R. B. A., Berndt, A., Gerrits, W. J. J., Dijkstra, J., van Zijderveld, S. M., Newbold, J, R., and Perdok, H. B. 2015. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci. 90:2317–2323.

Immig, I., Demeyer, D., Fiedler, D., Van Nevel, C., and Mbanzamihigo, L. 1996. Attempts to induce reductive acetogenesis into a sheep rumen. Arch. Tierernaehr. 49:363–370.

IPCC, 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex, P. M. Midgley, editors]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp., doi:10.1017/CBO9781107415324.

Ivan, M., Mir, P. S., Koening, K. M., Koening, L. M., Rode, L. M., Neill, L., Entz, T., and Mir, Z. 2001. Effects of dietary sunflower seed oil on rumen protozoa population and tissue concentration of conjugated linoleic acid in sheep. Small Rumin. Res. 41:215–227.

IPCC. 2006. Guidelines for National Greenhouse Gas Inventories. www.ipcc-nggip.iges.or.jp/public/2006gl. (Accessed 3 September 2014.).

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, L.A. Meyer, editors]. IPCC, Geneva, Switzerland, 151 pp.

Jalč, D., Baran, M., Vendrák, T. and Siroka, P. 1992. Effect of monensin on fermentation of hay and wheat bran investigated by the rumen simulation technique (RUSITEC) Arch. Tierernaehr. 42:147–152,

Janssen, P. H., and Kirs, M. 2008. Structure of the archaeal community of the rumen. Appl. Environ. Microbiol. 74: 3619–3625.

Janssen, P. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160:1–22.

Jayanegara, A., Ridla, M., Laconi, E., and Nahrowi. 2014. Can plant saponins lower methane emissions without hampering the nutrient digestibility of ruminants. Proceedings of the 16th AAAP Animal Science Congress Vol. II. 10-14 November 2014, Gadjah Mada University, Yogyakarta, Indonesia.

Johnson, E. D., Wood, A. S., Stone, J. B., and Moran Jr., E. T. 1972. Some effects of methane inhibition in ruminants (steers). Can. J. Anim. Sci. 52:703–712.

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

Karnati, S. K. R., Yu, Z., Firkins, J. L. 2008. Investigating unsaturated fat, monensin, or bromoethanesulfonate in continuous cultures retaining ruminal protozoa. II. Interaction of treatment and presence of protozoa on prokaryotic communities. J. Dairy Sci. 92:3861–3873.

Keltjens, J. T. and Vogels, G. D. 1996. Metabolic regulation in methanogenic Archaea during growth on hydrogen and CO_2. Environ. Monit. Assess. 42:19–37.

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

Knight, T., Ronimus, R. S., Dey, D., Tootill, C., Naylor, G., Evans, P., Molano, G., Smith, A., Tavendale, M., Pinares-Patiño, C. S., and Clark, H. 2011. Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. Anim. Feed Sci. Technol. 166–167:101–112.

Kumar, S., Choudhury, P. K., Carro, M. D., Griffith, G. W., Dagar, S. S., Puniya, M., Calabro, S., Ravella, S. R., Dhewa, T., Upadhyay, R. C., Sirohi, S. K., Kundu, S. S., Wanapat, M.

and Puniya, A. K. 2014. New aspects and strategies for methane mitigation from ruminants. Appl. Microbiol. Biotech. 98:31–44.

Lee, S. Y., Yang, S. H., Lee, W. S., Kim, H. S., Shin, D. E., and Ha, J. K., 2009. Effect of 2-bromoethanesulfonic acid on *in vitro* fermentation characteristics and methanogen population. Asian-Aust. J. Anim. Sci. 22:42–48.

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

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54:519–543.

Li, M., Penner, G. B., Hernandez-Sanabria, E., Oba, M., and Guan, L. L. 2009. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107:1924–1934.

Li, L., Davis, J., Nolan, J., and Hegarty, R. 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. Anim. Prod. Sci. 52:653–658.

Li, Y., He, M., Li, C., Forster, R., Beauchemin, K. A., and Yang, W. 2012. Effects of wheat dried distillers' grains with solubles and cinnamaldehyde on *in vitro* fermentation and protein degradation using the Rusitec technique. Arch. Anim. Nutr. 66:131–148.

Liu, Y., and Whitman, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125: 171–189.

Li, Y. L., Li, C., Beauchemin, K. A. and Yang, W. Z. 2013. Effects of a commercial blend of essential oils and monensin in a high-grain diet containing wheat distillers' grains on *in vitro* fermentation. Can. J. Anim. Sci. 93:387–398.

Lopez, S., McIntosh, F. M., Wallace, R. J., and Newbold, C. J. 1999. Effect of adding acetogenic bacteria on methane production by mixed rumen microorganisms. Anim. Feed Sci. Technol. 78:1–9.

Lovett, D., Lovell, S., Stack, L., Callana, J., Finlay, M., Conolly, J., and O'Mara, F. P. 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.

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

Martínez, M. E., Ranilla, M. J., Ramos, S., Tejido, M. L., Carro, M. D. 2009. Effects of dilution rate and retention time of concentrate on efficiency of microbial growth, methane production, and ruminal fermentation in Rusitec fermenters. J. Dairy Sci. 92:3930–3938.

Martinez-Fernandez, G., Arco, A., Abecia, L., Cantalapiedra-Hijar, G., Molina-Alcaide, E., Martin-Garcia, A. I., Kindermann, M., Duval, S., and Yañez-Ruiz, D. R. 2013. The addition of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol in the diet of sheep substantially reduces methane emissions and the effect persists over a month. Adv. Anim. Biosci. 4:368 (Abstr.).

Martínez-Fernández, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martín-García, A.I., Molina-Alcaide, E., Kindermann, M., Duval, S., and Yáñez-Ruiz, D.R.,2014. Effects of

ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. J. Dairy Sci. 97:3790–3799.

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

McBride, B. C., and Wolfe, R. S. 1971. A new coenzyme of methyl transfer, coenzyme M. Biochem.10:2317–2324.

McCrabb, G. J., Berger, K. T., Magner, T., May, C., and Hunter, R. A.1997. Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. Aust. J. Agric. Res. 48:323–329.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43:99–109.

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

Machmüller, A., Ossowski, D. A., Wanner, M., and Kreuzer, M. 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Anim. Feed Sci. Tech. 71:117–130.

Meale, S. J., Ding, S., He, M. L., Dugan, M. E. R., Ribeiro Jr., G. O., Alazzeh, A. Y., Holo, H., Harstad, O. M., McAllister, T. A., and Chaves, A.V. 2014. Effect of Propionibacterium freudenreichii on ruminal fermentation patterns, methane production and lipid biohydrogenation of beef finishing diets containing flaxseed oil in a rumen simulation technique. Can. J. Anim. Sci. 94:685–695.

Melendez, P., Goff, J. P., Risco, C. A., Archbald, L. F., Littell, R., and Donovan, G. A. 2004. Effect of a monensin controlled-release capsule on rumen and blood metabolites in Florida Holstein transition cows. J. Dairy Sci. 87:4182–4189.

Miller, T. L., and Lin, C. 2002. Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. Int. J. Syst. Evol. Micr. 52:819–822.

Mitsumori, M. and Sun, W. 2008. Control of rumen microbial fermentation for mitigating methane emissions from the rumen. Asian-Aust. J. Anim. Sci. 1(vol. 21):144-154.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., and McSweeney, C. S. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108:482–491.

Morgavi, D. P., Forano, E., Martin, C., and Newbold, C. J. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4(7):1024–1036.

Morrison, M., and Mackie, R. I. 1996. Nitrogen metabolism by ruminal microorganisms: Current understanding and future perspectives. Aust. J. Agric. Res. 47:227–246.

Munksgaard, L., Jensen, M. B., Pedersen, L. J., Hansen, S. W., and Matthews, L.. 2005. Quantifying behavioural priorities—Effects of time constraints on behaviour of dairy cows, *Bos taurus*. Appl. Anim. Behav. Sci. 92:3–14.

Nagaraja, T. G., Avery, T. B., Bartley, E. E., Galitzer, S. J., and Dayton, A. D. 1981. Prevention of lactic acidosis in cattle by lasalocid or monensin. J. Anim. Sci. 53:206–216.

Nanon, A., Suksombat, W., Beauchemin, K. A., and Yang, W. Z. 2014. Assessment of lemongrass oil supplementation in a dairy diet on *in vitro* ruminal fermentation characteristics using the rumen simulation technique. Can. J. Anim. Sci. 94:731–736.

Newbold, C. J., Lassalasl, B., and Jouany, J.P. 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production *in vitro*. Lett. Appl. Microbiol. 21:230–234.

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

Nollet, L., Demeyer, D., and Verstraete, W. 1997. Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. Appl. Environm. Microbiol. 63:194–200.

NRC. 1996. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Nutt, B. G., Holloway, J. W., and Butts, W. T. 1980. Relationship of rumen capacity of mature angus cows to body measurements, animal performance and forage consumption on pasture. J Anim. Sci. 51:1168–1176.

O'Brien, M., Navarro-Villa, A., Purcell, P. J., Boland, T. M., and O'Kiely, P. 2014. Reducing *in vitro* rumen methanogenesis for two contrasting diets using a series of inclusion rates of different additives. Anim. Product. Sci. 54:141–157.

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

Ogimoto, K., and Imai, S. 1981. Atlas of Rumen Microbiology. Japan Scientific Societies Press, Tokyo.

Patra, A. K. 2012. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ. Monit. Assess. 184:1929–1952.

Patra, A. K. 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. Livest. Sci.155:244–254.

Penner, G. B., Beauchemin, K. A., and Mutsvangwa, T. 2006. An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. J. Dairy Sci. 89:2132–2140.

Plaa, G.L. 2000. Chlorinated methanes and liver injury: highlights of the past 50 years. Annu. Rev. Pharm. Toxicol. 40:43–65.

Prakash, D. 2014. Methyl-coenzyme M reductase: Elucidating the process of activation and study of the effect of the methanogenesis inhibitor 3-nitrooxypropanol (Doctoral dissertation). Auburn University. https://etd.auburn.edu/handle/10415/4463.

Puchala, R., Min, B. R., Goetsch, A. L., and Sahlu, T. 2005. The effect of a condensed tannin–containing forage on methane emission in goats, J. Anim. Sci. 83:182–186.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S., and Steinberg, W. 2014. Effects of incremental rumen doses of 3-nitrooxypropanol onmethane production, digestion and rumen parameters in lactating dairy cows. J. Dairy Sci. 97, 3777–3789.

Rhine, E. D., Sims, G. K., Mulvaney, R. L., and Pratt, E. J. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Sci. Soc. Am. J. 62:473–480.

Robinson, J. A., Strayer, R. F., and Tiedje, J. M. 1981. Method for measuring dissolved hydrogen in anaerobic ecosystems: Application to the rumen. Appl. Environ. Microbiol. 41:545–548.

Romero-Perez, A., Beauchemin, K. A., Okine, E. K., and Duval, S. M. 2013. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). Adv. Anim. Biosci. 4:389 (Abstr.).

Romero-Perez A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682–4693.

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

Romero-Pérez, A., Okine, E. K., Guan, L. L., Duval, S. M., Kindermann, M., and Beauchemin, K. A. 2015b. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). Anim. Feed Sci. Technol. 209:98–109.

Russell, J. B. 2002. Rumen microbiology and its role on ruminant nutrition. J. B. Russell, Ithaca, NY.

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

Saengkerdsub, S., and Ricke, S. 2014. Ecology and characteristics of methanogenic archaea in animals and humans. Crit. Rev. Microbiol. 40:97–116.

Santoso, B., Mwenya, B., Sar, C., Gamo, Y., Kobayashi, T., Morikawa, R., Kimura, K., Mizukoshi, H., Takahashi, J. 2004. Effects of supplementing galacto-oligosaccharides, *Yucca schidigera* or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livest. Prod. Sci. 91:209–217.

Sauer, F. D., Fellner, V., Kinsman, R. Kramer, J. K. G., Jackson, H. A., Lee, A. J., and Chen, S. 1998. Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. J. Anim. Sci. 76:906–914.

Sauvant, D., and Giger-Reverdin, S. 2009. Modélisation des interactions digestives et de la production de méthane chez les ruminants. INRA Prod. Anim. 22:375–384.

Sawyer, M. S., Hoover, W. H., and Sniffen, C. J. 1974. Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. J. Anim. Sci. 38:908–914.

Seo, S., Tedeschi, L. O., Lanzas, C., Schwab, C. G., and Fox, D. G. 2006. Development and evaluation of empirical equations to predict feed passage rate in cattle. Anim. Feed Sci. Technol. 128:67–83.

Shima, S., and Thauer, R. K. 2005. Methyl-coenzyme M reductase and the anaerobic oxidation of methane in methanotrophic archaea. Curr. Opin. Microbiol. 8:643–648.

Śliwiński, B. J., Soliva, C. R., Machmüller, A., Kreuzer, M. 2002. Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation. Anim. Feed Sci. Technol. 101:101–114.

Soliva, C. R., Amelchanka, S. L., Duval, S. M., Kreuzer, M., 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). Br. J. Nutr. 106:114–122.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., and de Haan, C. 2006. Livestock's long shadow: Environmental issues and options. Food and Agriculture Organization of the United Nations, Rome, Italy.

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

St-Pierre, B., and Wright, A.-D. G. Diversity of gut methanogens in herbivorous animals. Animal 7:49–56.

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M., and Firkins, J. L. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. J. Nutr. 134:3378–3384.

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., and Sivakumaran, S. 2005. Methane production from *in vitro* rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis, Anim. Feed Sci. Technol. 123–124:403–419.

Taylor, C. D., and Wolfe, R. S. 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3$). J. Biol. Chem. 249:4879–4885.

Tekippe, J. A., Hristov, A. N., Heyler, K. S., Cassidy, T. W., Zheljazkov, V. D., Ferreira, J. F. S., Karnati, S. K., and Varga, G. A. 2011. Rumen fermentation and production effects of *Origanum vulgare* L. leaves in lactating dairy cows. J. Dairy Sci. 94:5065–5079.

Thauer, R. 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology 144:2377-2406.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W., and Hedderich R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nature Rev. Microbiol. 6:579–591.

Tomkins, N. W., Colegate, S. M., and Hunter, R. A. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49:1053–1058.

Trei, J. E., Parish, R. C., Singh, Y. K., and Scott, G. C. 1971. Effect of methane inhibitors on rumen metabolism and feedlot performance of sheep. J. Dairy Sci. 54:536 –539.

Ungerfeld, E. M., Rust, S. R., Boone, D. R., and Liu, Y. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. J. Appl. Microbiol. 97:520–526

Ungerfeld, E.M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6:1–17.

United Nations (UN). 2015. World Population Prospects: The 2015 Revision.

Van Nevel, C. J., Henderickx, H. K., Demeyer, D. I., and Martin, J. 1969. Effect of chloral hydrate on methane and propionic acid in the rumen. Appl. Microbiol. 17:695–700.

Van Nevel, C., and Demeyer, D. 1981. Effect of methane inhibitors on the metabolism of rumen microbes *in vitro*. Archiv für Tierernaehrung, 31(2):141–151.

Van Nevel, C., and Demeyer, D. 1995. Feed additives and other interventions for decreasing methane emissions. In Biotechnology in animal feeds and animal feeding [R. J. Wallace and A. Chesson, editors] VCH Verlagsgesellschaft mbH., Weinheim, Germany. p. 329–349.

Van Nevel, C.J., and Demeyer, D.I. 1996. Control of rumen methanogenesis. Environ. Monit. Assess. 42:73–97.

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. 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., Gerrits, W. J. J., Apajalahti, J. A. Newbold, J. R., Dijkstra, J., Leng, R. A., and Perdok, H. B. 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., Gerrits, W. J. J., Dijkstra, J., Newbold, J. R., Hulshof, R. B. A., and Perdok, H. B. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94:4028–4038.

Veira, D. M., Ivan, M., and Jui, P. Y. 1983. Rumen ciliate protozoa: effects on digestion in the stomach of sheep. J. Dairy Sci. 66:1015–1022.

Vogels G. D., W. F. Hoppe, and C. K. Stumm. 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ Microbiol. 40:608-612.

Waghorn, G. C., Clark, H., Taufa, V., and Cavanagh, A. 2008. Monensin controlled-release capsules for methane mitigation in pasture-fed dairy cows. Austr. J. Exp. Agric.48:65–68.

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

Wang, C. J., Wang S. P., and Zhou, H. 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Anim. Feed Sci. Technol. 148:157–166.

Wischer, G., Boguhn, J., Steingaß, H., Schollenberger, M., Hartung, K., and Rodehutscord, M. 2013. Effect of monensin on *in vitro* fermentation of silages and microbial protein synthesis. Arch. Anim. Nutr. 67:219–234.

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

Wood, J, M., Kennedy, F. S., and Wolfe, R. S. 1968. The reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. Biochem. 7:1707–1713.

Wood, T. A., Wallace, R. J. Rowe, A., Price, J., Yañez-Ruiz, D. R., Murray, P., and Newbold, C. J. 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Anim. Feed Sci. Technol. 152:62–71.

World Meteorological Organization (WMO). 2014. WMO Greenhouse Gas Bulletin: The State of Greenhouse Gases in the Atmosphere Based on Global Observations through 2013. 6 November 2014. No. 10. Available online at http://www.wmo.int/pages/prog/arep/gaw/ghg/GHGbulletin.html.

Zhang, D. F, and Yang, H. J. 2012. Combination effects of nitrocompounds, pyromellitic diimide, and 2-bromoethanesulfonate on *in vitro* ruminal methane production and fermentation of a grain-rich feed. J. Agric. Food Chem. 60:364–371.

Zhou, M., Hernandez-Sanabria, E., and Guan, L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Appl. Environ. Microbiol. 75:6524–6533.

Zhou, M., Chung, Y.-H., Beauchemin, K. A., Holtshausen, L., Oba, M., McAllister, T. A., and Guan, L. L. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. J. Appl. Microbiol. 111:1148–1158.

Zhou, Z., Meng, Q., and Yu, Z. 2011. Effects of methanogenic inhibitors on methane production and abundances of methanogens and cellulolytic bacteria in *in vitro* ruminal cultures. Appl. Environm. Microbiol. 77:2634–2639.

Zhou, M., Chen, Y., Haisan, J., Romero-Perez, A., Beauchemin, K. A., Oba. M., Kindermann, M., Duval, S., and Guan, L. L. 2015. Effects of feeding 3-nitrooxypropanol (NOP) on rumen microbial profiles in lactating dairy cows and beef cattle. Proceedings of the ADSA-ASAS Joint Annual Meeting, July 12-16, 2015, Orlando, Florida, USA.

Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R., and Stahl, D. A. 2000. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. Environ. Microbiol. 2:632–643.